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ISSUED MONTHLY FOR THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS BY CHARLES C THOMAS, PUBLISHER, SPRINGFIELD, ILLINOIS

Annual Subscription $11.00 Single Copies $1.25

The Laurentian Hormone Conference of the A.A.A.S. will hold its 1950 meeting at the Forest Hills Hotel, Franconia, New Hampshire, September 10th to 15th. The following program has been arranged:

I. GONAD DEVELOPMENT AND FUNCTION

A. Embryogenesis of the adrenal and the reproductive glands.
Dr. Emil Witschi, The State University of Iowa
Sunday Evening, September 10

B. Mammalian spermatogenesis.
Dr. Warren O. Nelson, The State University of Iowa
Monday Morning, September 11

C. Oogenesis in the mature ovary.
Dr. S. Zuckerman, University of Birmingham, England
Monday Morning, September 11

II. THE USE OF ISOTOPES IN HORMONE PROBLEMS

D. Studies in the metabolism of steroid hormones.
Dr. T. F. Gallagher, Sloan-Kettering Institute
Monday Evening, September 11

E. Biosynthesis of steroids.
Dr. Konrad Bloch, The University of Chicago
Tuesday Morning, September 12

III. HORMONES AND METABOLISM

F. Parameters of metabolic problems.
Dr. Dwight J. Ingle, The Upjohn Company
Tuesday Morning, September 12

G. Metabolic processes influenced by the growth hormone.
Dr. F. J. Young, Biochemical Laboratory, Cambridge, England
Tuesday Evening, September 12

H. The problem of adrenal androgens in man.
Dr. Allan T. Kenyon, The University of Chicago
Wednesday Morning, September 13

IV. CHEMISTRY, PHYSIOLOGY AND CLINICAL ENDOCRINOLOGY OF THE ADRENAL CORTEX

I. Chemistry of adrenal cortex steroids.
Dr. P. L. Julian, The Glidden Company
Wednesday Morning, September 13

J. The nature of adrenal secretory activity.
Dr. Oscar Hechter, The Worcester Foundation for Experimental Biology
Thursday Morning, September 14

K. The adrenal cortex in salt and water metabolism.
Dr. Robert Gaunt, Syracuse University
Thursday Morning, September 14

L. A consideration of the role of the adrenal cortex in the regulation of certain metabolic processes.
Dr. Frank L. Engel, Duke University
Thursday Evening, September 14

M. Observations on the physiological effects of cortisone and ACTH in man.
Drs. R. G. Sprague, M. H. Power and H. L. Mason, The Mayo Clinic
Friday Morning, September 15

V. HORMONE THERAPY IN CANCER

N. Experimental studies with ACTH and cortisone in patients with neoplasma.
Dr. O. H. Pearson, Sloan-Kettering Institute
Friday Morning, September 15

O. Steroid hormone therapy in cancer.
Dr. I. T. Nathanson, Massachusetts General Hospital
Friday Evening, September 15

Attendance at this Conference is limited by the accommodation available at the hotel, but the Committee on Arrangements invites applications for attendance from interested investigators and specialists in the hormone field. The Committee on Arrangements consists of R. W. Bates, E. R. Squibb & Sons; R. D. H. Heard, McGill University; A. D. Odell, Charles E. Frosst & Co.; E. C. Reifenstein, Jr., Sloan-Kettering Institute; A. White, School of Medicine, University of California at Los Angeles; and G. Pincus, Chairman, The Worcester Foundation for Experimental Biology. Application blanks for attendance at the Conference may be obtained from the Chairman at 222 Maple Avenue, Shrewsbury, Massachusetts. Applications to be considered by the Committee must be received by May 20, 1950.

ENDOCRINOLOGY

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WORK OUTPUT AND BLOOD GLUCOSE VALUES IN NORMAL AND IN DIABETIC RATS SUBJECTED TO THE STIMULATION OF MUSCLE

DWIGHT J. INGLE, JAMES E. NEZAMIS AND KATHRYN L. RICE

From the Research Laboratories, The Upjohn Company

KALAMAZOO, MICHIGAN

IT WAS previously shown (Ingle, 1948; Ingle and Nezamis, 1948) that muscular work accelerates the rate of fall of blood glucose in the eviscerate rat and increases the tolerance of the eviscerate rat for intravenously administered glucose. The eviscerate rat is insulin deficient. The present experiments involve normal and depancreatized rats which did not receive glucose or insulin. The stimulation of muscle caused a rapid fall in the level of blood glucose to hypoglycemic levels in both normal and in diabetic rats, but to lower and more frequently fatal levels in the normals. The output of work in those animals which survived was just as high in the diabetic animals as in the normals.

METHODS

Male rats of the Sprague-Dawley strain were depancreatized by the method of Ingle and Griffith (1942) at a weight of approximately 300 gms. The pancreas was removed as completely as possible. The stock diet was Archer Dog Pellets. After the animals had recovered from the operation, they were placed in metabolism cages and given a fluid, medium carbohydrate diet (Table 1) to eat in amounts of 13 cc. each morning and late afternoon. After the animals were adapted to the cages, 24-hour samples of urine were collected each morning prior to feeding. The determination of urinary glucose was according to Benedict (1911). Those diabetic rats which ate their food promptly, excreted an average of 5 grams or more of urinary glucose during the last 3 days of observation and which appeared to be vigorous, were selected for the stimulation of muscle. Normal rats, matched for weight with the diabetic animals, were maintained in the metabolism cages under the same conditions of diet prior to being used for

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the work test. Approximately 16 hours lapsed between the last feeding and the beginning of work.

The animals were prepared for the stimulation of muscle according to Ingle (1944) with the three following modifications. First, a Nerve Stimulator (Model B, Upjohn) was used to stimulate muscle at the rate of 5 times per second. Second, the duration of each pulse was 20 msec. and the intensity was 20 ma. Third, the stimulus was passed from the right back foot

TABLE 1. MEDIUM CARBOHYDRATE DIET

Constituent	g	Mg.
Cellu flour (Chicago Dietetic Supply)	120	
Osborne & Mendel salt mixture	40	
Dried yeast (Pabst)	100	
Wheat germ oil	10	
Cod liver oil	10	
Vit. K (2-methyl-1,4-naphthoquinone)		100
Mazola oil	200	
Casein (Labco)	160	
Starch	200	
Dextrin	190	
Sucrose	200	
Water to make total of	2000 cc	

to the left back foot thereby activating the entire musculature of both legs. The left leg was weighted with 100 gms., and the distance the weight was lifted was registered on automatic work recorders. Stimulation was continued until the death of the animal or for 72 hours. Each animal received 20 cc. of 0.85 per cent sodium chloride solution by continuous subcutaneous injection each 24 hours. The animals were enclosed in a cabinet with temperature constant at $28 \pm 0.5°C$. Rates of work were noted, and samples of tail blood were taken at the beginning of stimulation, 6, 24, 48 and 72 hours later. The analyses of blood glucose were by the method of Miller and Van Slyke (1936).

EXPERIMENTS AND RESULTS

The diabetic rats were divided into 3 groups based upon the level of urinary glucose. The rats of one group excreted from 5 to 6 grams of glucose daily, the second group from 6 to 7 grams daily, and the third group more than 7 grams daily. It was determined to test normal and diabetic rats until 10 animals for each of 4 groups had survived the stimulation of muscle for 72 hours. All of the data are summarized in Table 2. Many of the normal rats died in hypoglycemic

TABLE 2. RATES OF WORK (RPM)* AND BLOOD GLUCOSE VALUES (BS) FOR NORMAL AND DIABETIC RATS. AVERAGES AND RANGE

Urine glucose mgm./day	Initial			6 hours			24 hours			48 hours			72 hours		
	N	RPM	BS	N	RPM	BS	N	RPM	BS	N	RPM	BS	N	RPM	BS
0 (normal)	42	63 (45–86)	129 (98–150)	29	28 (10–42)	33 (21–51)	22	16 (2–21)	37 (26–66)	13	3 (0–6)	27 (20–39)	10	1 (0–4)	35 (29–47)
5563 (5128–5994)	12	56 (24–79)	296 (218–434)	11	38 (30–44)	50 (21–75)	11	16 (12–32)	47 (33–62)	11	5 (0–11)	46 (35–72)	10	2 (0–6)	47 (33–75)
6433 (6017–6906)	14	52 (32–72)	360 (171–465)	14	36 (26–44)	59 (45–90)	14	13 (3–24)	54 (44–80)	14	4 (0–7)	56 (33–108)	10	1 (0–4)	53 (24–90)
7637 (7094–9188)	18	55 (34–76)	364 (219–440)	18	41 (26–53)	64 (45–95)	15	14 (0–21)	68 (35–98)	11	6 (3–9)	66 (42–105)	10	2 (0–5)	55 (30–98)

* Recorder revolutions per minute. Each recorder revolution represents approximately 400 gram-centimeters of work.

convulsions within the first few hours. The diabetic rats were hyperglycemic at the beginning of work, and although the blood glucose dropped precipitously, only a few animals died with hypoglycemic convulsions. As noted in Table 2, 42 normal starters were required to complete a series of 10 survivors of 72 hours of stimulation as compared to a requirement of 12 starters for the 5 grams plus, 14 starters for the 6 grams plus, and 18 starters for the 7 grams plus.

Although the initial average rates of work were less than normal in each of the 3 groups of diabetic rats, the latter animals showed better maintenance of work output during the early hours of muscle stimulation. The average total work output of the diabetic rats was greater than that of normal rats due in part to the higher mortality

TABLE 3. RATES OF WORK (RPM)* AND BLOOD GLUCOSE VALUES (BS) FOR NORMAL AND DIABETIC RATS WHICH SURVIVED FOR 72 HOURS. AVERAGES AND RANGE FOR 10 RATS PER GROUP

Urine glucose	Initial		6 hours		24 hours		48 hours		72 hours	
mgm./day	RPM	BS	RPM	BS	RPM	BS	RPM	BS	RPM	BS
normal	62 (45–82)	128 (110–161)	30 (19–42)	33 (24–51)	16 (10–21)	35 (27–48)	3 (0–6)	27 (21–39)	1 (0–4)	35 (23–47)
5592 (5101–5994)	53 (24–79)	298 (218–434)	38 (30–44)	52 (41–75)	16 (7–32)	47 (33–62)	5 (0–11)	45 (35–72)	2 (0–6)	47 (33–75)
6444 (6017–6906)	51 (32–72)	343 (171–444)	35 (26–44)	57 (45–90)	14 (8–24)	50 (30–69)	4 (0–7)	51 (32–71)	1 (0–4)	53 (24–90)
7408 (7094–9188)	56 (34–76)	343 (219–440)	40 (26–53)	63 (45–83)	15 (7–21)	67 (42–98)	6 (3–9)	66 (42–105)	2 (0–5)	55 (30–98)

* Recorder revolutions per minute. Each recorder revolution represents approximately 400 gram-centimeters of work.

among the normals. Because of this difference in incidence of survival, the data for all of the rats which lived for 72 hours in each of the 4 groups are summarized in Table 3. The rates of work for the diabetic rats were equal or superior to those of normal animals. The total average number of recorder revolutions for each group was as follows: normals, 53,919; 5 gms. plus, 63,570; 6 gms. plus, 53,414; 7 gms. plus, 61,271.

As shown in both Tables 2 and 3, the animals of all 4 groups developed hypoglycemia during work, but the values for blood glucose were higher in the diabetic series and were directly related to the level of urinary glucose.

DISCUSSION

Under the conditions of these experiments, rats made severely diabetic by pancreatectomy were able to survive the intensive stimulation of muscle better than normal rats, and the work output of the survivors was as good or better than the performance of the normal survivors. It is apparent that severe insulin deficiency in the rat is compatible with vigorous contraction of muscle. These animals were as completely depancreatized as we were able to prepare, and the 6 and 7 grams plus series were excreting amounts of glucose greater than their dietary carbohydrate. Nevertheless, there may have been physiologically important amounts of insulin present.

Vigorous muscle work can cause a precipitous fall in the level of blood glucose in the severely diabetic rat, and in a few instances brought death from hypoglycemic convulsions. Fasting for 72 hours without work causes little if any fall in the level of blood glucose in either normal or diabetic rats (Ingle, unpublished data). Normal rats which are anesthetized, restrained and fasted for 72 hours without work do not develop hypoglycemia (Ingle, Nezamis and Morley In press). From these and earlier observations on the eviscerate rat (Ingle, 1948; Ingle and Nezamis, 1948), it is tempting to conclude that the contracting muscle can utilize glucose for energy purposes without insulin. However, we do not know the actual fate of the glucose, although its disappearance from the blood was correlated with muscle work, and we do not know that any of our rats from this or earlier experiments were completely deficient in insulin.

In these experiments, a number of factors of selection were operating in the setting up of the 3 groups of diabetic rats. About 10 per cent of our extensively depancreatized rats died from the immediate effects of the operation. Approximately 80 per cent of the remaining animals either died in diabetic coma or were discarded because they were "weak and ill." It is quite possible that the most completely insulin deficient animals were eliminated by these selective factors. On the other hand, none of the rats which were eliminated by death or by overt weakness showed more severe glycosuria than the rats of the 7 gms. plus series.

It is generally believed by students of clinical diabetes that the carbohydrate tolerance of the diabetic patient is improved by exercise when some insulin is present, but that the severely diabetic patient without insulin is not improved by exercise. Diabetic rats that are already overtly "weak and sick" collapse quickly as the result of the stimulation of muscle, and the administration of insulin at this time does not improve their response. Work, under the conditions of these experiments, is a severe stress and will cause the rapid collapse of an animal debilitated by any set of causes. We are not aware of adequate studies of the effects of intensive long-continued exercise on severely diabetic patients not already debilitated by their disease.

The ideal experiment would involve tracing the utilization of tagged glucose administered by continuous intravenous injection to non-debilitated animals free of endogenous insulin with and without prolonged intensive stimulation of muscle and with and without exogenous insulin. If the conditions "insulin-free" and "non-debilitated" are not mutually exclusive, such an experiment should be possible of realization.

SUMMARY

Normal and diabetic male rats were anesthetized with sodium phenobarbital and were subjected to the faradic stimulation of both

back legs at a rate of 5 times per second until the death of the animal or for 72 hours. No glucose or insulin was administered. The stimulation of muscle caused a rapid fall in the level of blood glucose in both normal and diabetic rats, but to lower and more frequently fatal levels in the normals. The output of work was just as high in the diabetic animals which survived as in the normals.

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THE EFFECT OF CORTISONE ACETATE UPON THE GROWTH OF THE WALKER RAT CARCINOMA AND UPON URINARY NON-PROTEIN NITROGEN, SODIUM, CHLORIDE AND POTASSIUM

DWIGHT J. INGLE, MILDRED C. PRESTRUD AND KATHRYN L. RICE

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FOLLOWING the observations that large doses of cortisone (17-hydroxy-11-dehydrocorticosterone) retard the growth of the young rat (Wells and Kendall, 1940; Ingle, 1940) this compound has been found to retard the growth of a number of normal and malignant tissues. (Baker and Whitaker, 1948; Baker and Whitaker, 1949; Heilman and Kendall, 1944; Pearson *et al.*, 1949; Ragan, *et al.*, 1949).

In the present experiments, cortisone was found to retard the growth of the Walker rat carcinoma 256 in the force-fed rat. In confirmation of earlier studies (Ingle, Sheppard, Evans and Kuizenga, 1945), there was an accompanying glycosuria, loss of body-weight, rise in urinary non-protein nitrogen and potassium, and a temporary increase in the loss of sodium and chloride.

METHODS

Twenty male rats of the Sprague-Dawley strain having an initial weight of approximately 300 gms. were used in these experiments. The animals were placed in metabolism cages and fed a medium carbohydrate diet (Ingle, 1949). All of the rats were force-fed by stomach tube each morning (8:30 to 9:15 A.M.) and afternoon (4:15 to 5:00 P.M.). The techniques and diet were modifications of those described by Reinecke, Ball and Samuels (1939). The experiments were carried out in an air-conditioned room at a temperature of 74 to 78 degrees F. Twenty-four-hour samples of urine were collected at the same hour (8:00 to 8:30 A.M.) and were preserved with thymol and citric acid (1 gm. per sample) to insure the acidity of the samples for nitrogen analyses. The following methods of analysis were used: urine glucose, Benedict (1911); urinary chloride, the modified Volhard-Harvey titration as described by Peters and Van Slyke (1932); and urinary sodium and potassium by the flame photometer (Model 52A) as described by Overman and Davis (1947). The determination of non-protein nitrogen was by the micro-Kjeldahl procedure. Proteins were precipitated as the salts of tungstic acid by the Folin-Wu procedure; the organic matter was oxidized by sulfuric acid and hydrogen peroxide; the ammonia was distilled off into a standard acid solution and titrated with standard base.

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Following a control period of 14 days, each of the 20 rats received 0.1 cc. of a saline suspension of the tumor (Walker rat carcinoma 256) by subcutaneous injection high on the right flank. The animals were not anesthetized. Asepsis was successfully maintained during the preparation and implantation of the suspension of finely ground tumor tissue. In our experience, this procedure has been uniformly successful. The day following the implantation of the tumors, 10 of the rats were given 5 mgms. of cortisone acetate (Merck) per day for 10 days. The steroid was suspended in peanut oil, 5 mgms. per cc., and was given in divided subcutaneous injections each morning and late afternoon. The 10 control animals received peanut oil only, with the exception that on the 5th day of injection the control rats each received a single injection of 2.5 mgms. of cortisone acetate by error. At the end of 10 days of injections, all of the animals were subjected to necropsy. The tumors were weighed and measured, and samples of various tissues were fixed and sent to Dr. B. L. Baker, Department of Anatomy, University of Michigan School of Medicine for histochemical studies.

TABLE 1. THE EFFECT OF CORTISONE ACETATE UPON TUMOR WEIGHTS (GRAMS) AT THE END OF 10 DAYS OF TREATMENT. AVERAGES AND RANGE. TEN RATS PER GROUP

Cortisone acetate 5 mg. daily	Controls oil only
3.11 (1.5–6.69)	8.72 (6.2–12.5)

RESULTS

Tumor-size (*Table 1*) The tumors grew in all of the animals but at a retarded rate in those animals which received cortisone acetate.

Body weight (*fig. 1.*) All of the rats gained weight during the control periods. The rats which received cortisone acetate began to lose weight immediately and lost an average of 20 gms. during the injection period.

Urinary-nitrogen (*fig. 1.*) All of the rats remained in positive nitrogen balance during the control periods. The administration of cortisone acetate caused an immediate rise in urinary non-protein nitrogen so that by the 4th day and thereafter, these rats excreted more nitrogen in the urine than was contained in the diet.

Urinary-glucose (*fig. 1.*) None of the rats excreted glucose during the control periods, but all of the rats given cortisone acetate exhibited some degree of glycosuria, slight and temporary in some and severe and sustained in others.

Urinary potassium (*fig. 2.*) The administration of cortisone acetate caused an immediate increase in potassium excretion which roughly paralleled the changes in urinary nitrogen.

Urinary sodium and chloride (*fig. 2.*) The administration of cortisone acetate caused an immediate rise in the level of both sodium and chloride which was not sustained but gradually decreased to normal values by the end of the experiment. In the control series which re-

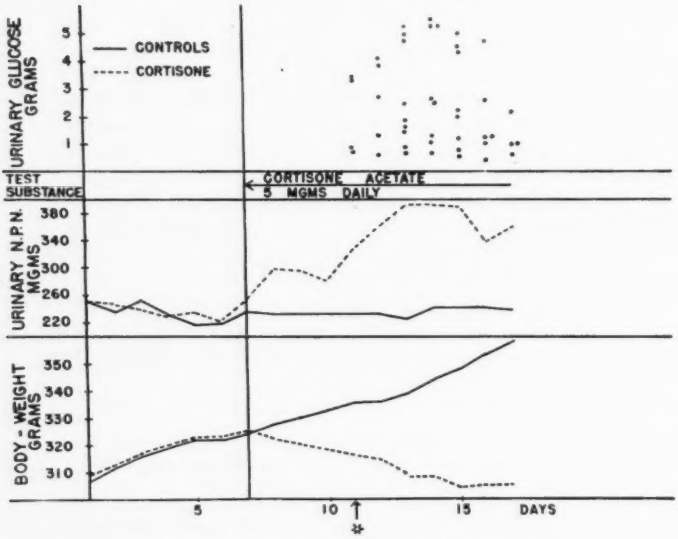

FIG. 1. The effect of cortisone acetate upon body-weight and urinary non-protein nitrogen (Averages) and individual values for urinary glucose. Ten rats per group. * 2.5 mg. of cortisone by error on this day.

ceived 2.5 mgms. of cortisone acetate by error on one day, there was an immediate rise in the loss of sodium and chloride followed by lower than normal excretion of these ions.

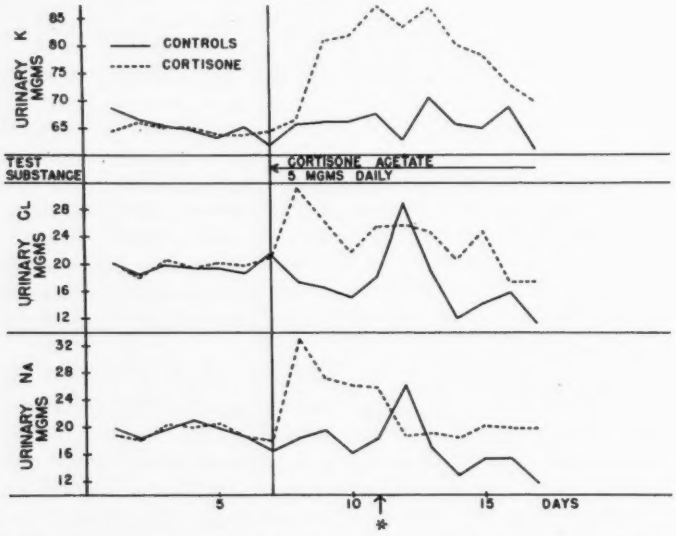

FIG. 2. The effect of cortisone acetate upon urinary sodium, chlorides and potassium. Averages of 10 rats per group. * 2.5 mg. of cortisone by error on this day.

DISCUSSION

The rate of growth of the tumor was definitely suppressed by the administration of cortisone acetate. This result may be without special significance since the dose of cortisone acetate was sufficiently large to cause a negative nitrogen balance. It may be of greater significance that the tumors were able to remain in an anabolic phase and gain in mass during a time that each animal was in negative nitrogen balance.

The general effects of cortisone acetate upon the urinary excretion of electrolytes were similar to the effects of cortisone and of 17-hydroxycorticosterone reported earlier (Ingle, Sheppard, Evans and Kuizenga, 1945), although there may have been a tendency for the rise of sodium and chloride excretion to be sustained for a longer time in the present study than was noted for the non-esterified cortisone. The peak of sodium and chloride loss was attained before any of the animals developed glycosuria and before they attained a peak of nitrogen excretion.

SUMMARY

Twenty intact male rats of the Sprague-Dawley strain having an initial weight of approximately 300 gms. were force-fed a medium carbohydrate diet. All of the rats were implanted with Walker carcinomas and 10 of them were given 5 mgms. of cortisone acetate (subcutaneous) daily for 10 days. The administration of the sterol caused a loss of body-weight, a rise of urinary non-protein nitrogen and potassium and a temporary rise in urinary sodium and chloride. The tumors gained in mass during the administration of cortisone acetate although the hosts were in negative nitrogen balance, but the extent of growth during 10 days was definitely suppressed below that of the controls.

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METABOLISM OF THE STEROID HORMONES: ISOLATION OF 13 STEROID METABOLITES FROM A PATIENT WITH (PROBABLE) ADRENAL HYPERPLASIA[1]

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THIS communication is a more complete report on the steroid metabolites of a patient, from whose urine the isolation of Δ?-androstenol -3(α)-one-17 (Dorfman *et al.*, 1945), androstanediol-3(α), 11(β)-one-17 (Miller *et al.*, 1945), and etiocholanediol-3(α), 17(α) (Miller and Dorfman, 1948), has been previously reported. The presence of a compound carrying oxygen at C_{11}, in high concentration, was chemical evidence for adrenal involvement, and further studies were carried out to determine the nature and amount of other urinary steroids present.

The results are in agreement with the findings characteristic of adrenal cortical hyperplasia: an increased excretion of 3(α)hydroxy steroids, carrying labels of the adrenal cortical hormones (oxygen substitution at C_{11} and C_{17} of the steroid nucleus), together with a slight or negligible increase in excretion of 3(β)hydroxy steroids which are characteristically predominant in adrenal cortical carcinoma.

The clinical picture of this patient (Wisconsin General Hospital No. 127483), who presented symptoms of virilism, has been described previously (Dorfman, *et al.*, 1945).

The 2 compounds occurring to the greatest extent, both of which carry adrenal cortical labels and which together represent 70% of the weight of purified crystalline material isolated from this patient, were androstanediol-3(α), 11(β)-one-17 (10 mg./liter) and pregnanetriol -3(α), 17, 20 (8 mg./liter). A summary of the 13 compounds obtained and their concentrations is given in Table 1.

Evidence is presented for the position of the double bond at C_9 in the Δ?-androstenol-3(α)-one-17 isolated from hydrolyzed urine of this patient. This compound is shown to be a probable artifact of dehydration due to acid hydrolysis derived from androstanediol-3(α), 11(β)-one-17.

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[1] Supported in part by a grant from the Committee on Endocrinology of the National Research Council, and in part by a grant (RG 1050) from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

TABLE 1. 13 STEROID METABOLITES ISOLATED FROM A PATIENT WITH (PROBABLE) ADRENAL CORTICAL HYPERPLASIA

Compound	Mg./liter
Androstanediol-3(α),11(β)-one-17 (11-Hydroxyandrosterone)	10
Pregnanetriol-3(α),17,20	8
Pregnanediol-3(α),20(α)	3
?17a-methyl-D-homoetiocholanediol-3(α),17a-one-17 (Rearrangement isomer of pregnanediol-3(α), 17-one-20)	1
Androsterone	1
Etiocholanol-3(α)-one-17	1
Δ^9-Androstenol-3(α)-one-17	1
3-chloro-Δ^5-androstenone-17	0.5
Δ^5-Pregnenediol-3(β),20(α)	0.2
Dehydroisoandrosterone	0.1
Etiocholanediol-3(α),17(α)	0.03
Allopregnanediol-3(α),20(α)	0.02
Pregnanediol-3(α),17-one-20	0.01

EXPERIMENTAL[2]

The unhydrolyzed urine was extracted with benzene, and the residual urine extracted with benzene after acid hydrolysis. These 2 extracts were examined separately.

UNHYDROLYZED URINE

76.1 liters of urine preserved with toluene were extracted in weekly batches between 3 and 9 days after collections were made. Each batch was extracted with benzene (3 times with $\frac{1}{4}$ volume). *Fractionation.* The fractionation procedure is shown in Fig. 1. The combined extracts were evaporated, taken up in 750 cc. ether and washed 4 times with 75 cc. 10% sodium hydroxide and water. Evaporation of the ether yielded the neutral fraction (14.2 gm.). This fraction was treated with 18 gm. of the Girard-Sandulesco reagent T at room temperature according to the method of Reichstein (1936). By this method, 4 fractions were separated: fraction A was extracted with ether from alkaline solution, fraction B was extracted from solution at pH 7, fraction C at pH 4.5, and finally fraction D was extracted with ether from the solution which had been strongly acidified with concentrated hydrochloric acid (1 volume of acid to 10 volumes of solution at pH 4.5). These fractions were further fractionated as indicated in Fig. 1.

NON-KETONIC FRACTION

Fraction A. This fraction (7.5 gm.) contained the non-ketones as well as the ketones which do not react at room temperature with the Girard reagent. Therefore, a second Girard separation was carried out at reflux temperature to remove the remaining ketones. This procedure yielded a ketonic fraction (about 200 mg.) and a non-ketonic fraction (4.8 gm.). The loss of material during this step probably represents organic acids and phenols. This ketonic fraction was not investigated. During evaporation of the ether solution containing the non-ketonic compounds, 1.6 gm. of crystalline material separated out. After removal of these crystals, evaporation of

[2] Melting points were determined with the Fisher-Johns apparatus and are uncorrected.

the ether solution yielded 3.2 gm. pink oil. The crystals and oil were purified by 3 main chromatograms as follows: *Chromatogram 1* ("Insoluble acetate"): 1.4 gm. of the crystals which precipitated out were acetylated (1.3 gm.). *Chromatogram 2* ("Insoluble free"): 0.27 gm. of crystals which precipitated out were chromatographed directly. *Chromatogram 3* ("Soluble acetate"): the pink oil was separated with digitonin yielding an α fraction (2.5 gm.) which was acetylated (2.7 gm.), chromatographed and rechromatographed as illustrated in Chromatogram 3.

Pregnanetriol-3(α), 17,20. This compound or its acetate was found in each of the 3 fractions, but by far the largest part in the ether insoluble material. Since the free compound was found to be very much more easily crystallized than the acetate, all fractions from Chromatograms 1, 2 and 3 containing this compound were combined, saponified, crystallized, and the

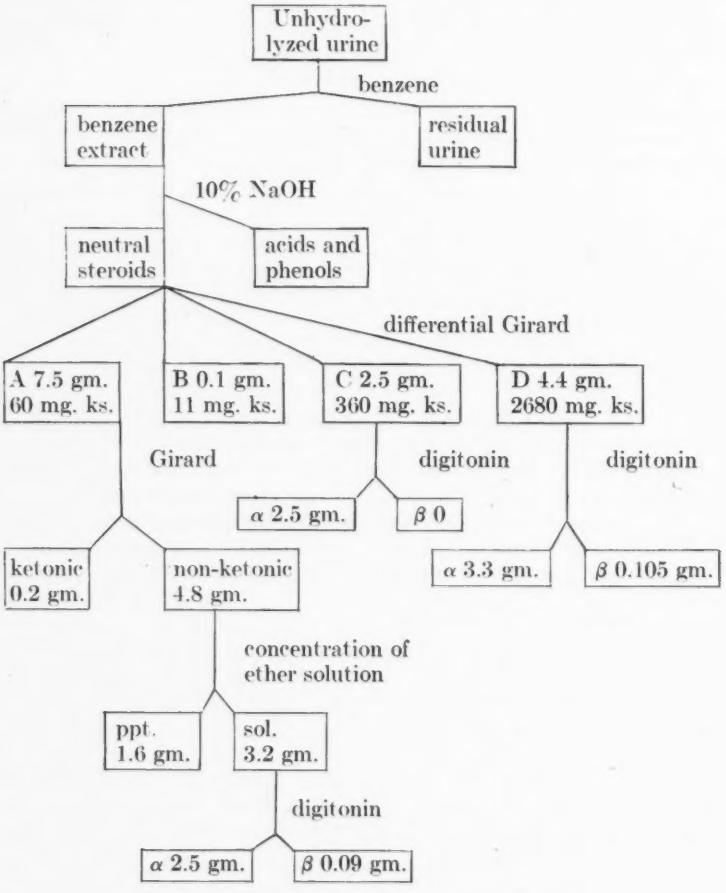

FIG. 1. Fractionation of Unhydrolyzed Urine

mother liquors rechromatographed, yielding 620 mg. crystals, m.p. 248.5–251°. The diacetate melted at 158–160°. Mason and Kepler (1945) reported m.p. 250–252° for the triol, and m.p. 156–158° for the diacetate. The free triol and its diacetate were crystallized from absolute acetone and dried over P_2O_5 at 56° and 1 micron pressure for 2 hours and analyzed.[3]

Analysis—$C_{21}H_{36}O_3$ (free compound)	Calculated	C 74.95, H 10.79
	Found	C 74.80, H 10.88
$C_{25}H_{40}O_5$ (diacetate)	Calculated	C 71.40, H 9.58
	Found	C 71.73, H 9.70

CHROMATOGRAM 1 ("Insoluble acetate")

Fraction	*Eluant* (80 cc./fraction)	*Compound*
2– 3	benzene-pet. ether 2:1	Pregnanediol-3(α),20(α) diacetate
4–17	benzene-pet. ether 2:1	Pregnanetriol-3(α),17,20 diacetate
18–23	benzene-pet. ether 3:1	Mixture
	benzene	Mixture
	benzene-ether 7:1	Mixture
24	benzene-ether 3:1	m.p. 222–224°
25–29	benzene-ether 3:1	Mixture
30–38	benzene-ether 1:3	m.p. 203–207°
39–42	benzene-ether 1:3	Mixture
	ether	Mixture
	ether-ethanol 1%	Mixture
43–50	ether-ethanol 5%	Pregnanetriol-3(α),17,20

CHROMATOGRAM 2 ("Insoluble free")

Fraction	*Eluant* (100 cc./fraction)	*Compound*
8–13	benzene-ether	Pregnanediol-3(α),20(α)
14–21	ether-ethanol 0.5%	Mixture
22–30	ether-ethanol 1–2.5%	Pregnanetriol-3(α),17,20
31–38	ether-ethanol 5–25%	Mixture
39–42	ether-ethanol 50%	m.p. 234–237°

Pregnanediol-3(α), 20(α). Most of this material also appeared in the ether-insoluble fractions. 54 mg. of the diacetate, m.p. 179–180°, were obtained from fraction 1 of Chromatogram 1. From fraction 2, 103 mg., m.p. 157–159°, were obtained (this lower melting point is also characteristic of pregnanediol-3(α),20(α) diacetate). This compound was also obtained from Chromatograms 2 and 3, and was identified by mixture with an authentic specimen.

Etiocholanediol-3(α),17(α). About 3 mg., m.p. 232–233°, were obtained from Chromatogram 3 ("Soluble acetate"). We have described this compound previously (Miller and Dorfman, 1948).

Allopregnanediol-3(α),20(α). About 2 mg., m.p. 240–241°, were obtained by rechromatographing fractions 6–21 from Chromatogram 3. When mixed with an authentic specimen, m.p. 239–240°, the melting point was 239.5–241°.

Unidentified Unsaturated Compound, m.p. 144–144.5°. This compound was obtained only from Chromatogram 3, in fractions 3–5 eluted with benzene-petroleum ether 1:2. Analysis of the acetate, m.p. 134°, which was crystallized from absolute methanol and dried over P_2O_5 for 1 hour at 56° and 1 micron pressure, indicated the formula $C_{23}H_{36}O_2$.

[3] The microanalyses reported in this paper were performed by Arlington Laboratories, Fairfax, Va.

Analysis—$C_{23}H_{36}O_2$	Calculated	C 80.18, H 10.53
	Found	C 80.09, H 10.62

Saponification yielded a product, m.p. 144–144.5°, which after reacetylation melted at 132–133°. When the latter was mixed with the isolated acetate, m.p. 134°, the melting point was 132.5–133.5°. This compound reduced bromine and was Beilstein negative. Chromic acid oxidation of the free compound, m.p. 144–144.5°, produced an unsaturated ketone, m.p. 144.5–145°; when mixed with the starting material, m.p. 144–144.5°, the melting point was 108–125°. Analysis of this ketone dried over P_2O_5 for 1 hour at 56° and 1 micron pressure indicated the possible formula $C_{21}H_{32}O$.

Analysis—$C_{21}H_{32}O$	Calculated	C 83.94, H 10.74
	Found	C 83.97, H 11.47

An attempt was made to identify this unsaturated compound, m.p. 144–144.5°, acetate m.p. 134°, which produced an unsaturated ketone, m.p. 144.5–145°, on oxidation. On the basis of the melting point, Δ^{17}-pregnenol-3(α) was excluded (Hirschmann, 1941), and the oxidation product of this compound was not $\Delta^{2 \text{ or } 3}$-allopregnenone-20 –Lieberman *et al.*, 1948). It was considered possible that the double bond was at C_2, C_3, C_{17} or C_{20} in a pregnane derivative, and that dehydration of the hydroxyl at C_3 or C_{20} of pregnanediol might produce the unknown compound. $\Delta^{2 \text{ or } 3}$-pregnenol-20 acetate, m.p. 120–122°, was prepared, but the compound was not this substance.

Other Unidentified Compounds. 9 other unidentified compounds (material of sharp melting point) were isolated from the non-ketonic fraction (see Chromatograms 1, 2 and 3). The amounts were too small in most cases for characterization. Only 1 compound, m.p. 225–226°, obtained from fraction 51 of Chromatogram 3, was analyzed. The analysis indicated 2 possible structures: $C_{25}H_{38}O_6$ (more likely) or $C_{21}H_{32}O_5$.

Analysis—$C_{25}H_{38}O_6$	Calculated	C 69.09, H 8.82
$C_{21}H_{32}O_5$	Calculated	C 69.20, H 8.85
	Found	C 69.09, H 8.86

This compound is an acetate (or non-acetylable) since acetylation produced no change in melting point, and no depression of melting point when the acetylated material was mixed with the starting material.

CHROMATOGRAM 3 ("Soluble acetate" from Fraction A)

Fraction* (From 1st chromato-gram)	Fraction† (From 2nd chromato-grams)	Fraction‡ (From 3rd chromato-grams)	Eluant	Compound
1–2			benzene-pet. ether	0
3–5			benzene-pet. ether	m.p. 134° (acetate)
6–21	12		benzene-alc. 0.1%	allopregnanediol-3(α),20(α)
	16–19		benzene-alc. 0.1%	etiocholanediol-3(α),17(α)
	23–29§	6–9	benzene-pet. ether	pregnanediol-3(α),20(α) diacetate
	33		benzene-alc. 2.5%	m.p. 226–227° (free)
22–27			benzene-pet. ether	oil
29			benzene-pet. ether	m.p. 189–192° (acetate)
28–50§	15		ether-alc. 1.0%	m.p. 223.5–225° (free)
	7–17§	12–14	benzene	m.p. 231–232° (acetate)
	18–21§	4–12	benzene-pet. ether	pregnanetriol-3(α),17,20 diacetate
		16	benzene-ether 10%	m.p. 134–136° (acetate)
51			benzene-ether 20%	m.p. 225–226° (acetate)

* Acetylated material.
† Saponified material.
‡ Reacetylated material.
§ These fractions were rechromatographed, yielding fractions as shown in the adjacent column. Only those fractions from the rechromatograms containing identified compounds or sharply melting material are given.

KETONIC FRACTIONS

Fraction B. This fraction (157 mg. containing 11 mg. 17-ketosteroids) was acetylated and chromatographed, yielding some unidentified crystalline material.

Fraction C. This fraction (2.5 gm. containing 360 mg. 17-ketosteroids) was acetylated, dissolved in 96 cc. benzene-petroleum ether 1:3, and put on a column of 40 gm. aluminum oxide (Chromatogram 4). At this point, the material began to crystallize; addition of 50 cc. benzene returned the material to solution.

CHROMATOGRAM 4 (Fraction C, acetylated)

Fraction	*Eluant* (100 cc./fraction)	*M.P.*
1	Benzene-pet. ether 1:1	155–156°
2– 6	benzene	oil (rechromatographed)
7–30	benzene	186–187°
	0.1% ethanol in benzene	186–187°
31–37	0.5% ethanol in benzene	mixture (rechromatographed)
38–44	benzene-ether	trace
	ether-acetone 3:1	oil
45	ether-acetone 1:1	235–237°

Fraction 1 yielded crystals, m.p. 155–156°, which were not identified.

Fractions 2–6 (461 mg. oil containing 112 mg. 17-ketosteroids) were saponified, separated with the Girard reagent a second time, reacetylated and rechromatographed, yielding 26.8 mg. crystals. After recrystallization, the melting point was 163°; mixture with an authentic specimen of androsterone acetate did not depress the melting point.

Fractions 7–30 yielded more than 100 mg. of a compound, m.p. 186–187°. A sample was dried over P_2O_5 at 100° and 1 micron pressure for 1 hour and analyzed.

Analysis—$C_{23}H_{36}O_4$	Calculated	C 73.36, H 9.64
	Found	C 73.96, H 9.96

Saponification yielded a product, m.p. 169°. A sample was dried over P_2O_5 at 100° and 1 micron pressure for 1 hour and analyzed.

Analysis—$C_{21}H_{34}O_3$	Calculated	C 75.40, H 10.29
	Found	C 74.82, H 9.98

After reacetylation of the saponified product, the melting point was 182.5–185° and mixture with the isolated compound did not depress the melting point. The oxime was prepared. 20 mg. of free compound, m.p. 169°, were dissolved in 0.2 cc. absolute ethanol and 0.2 cc. pyridine containing 16 mg. hydroxylamine hydrochloride, and heated on the water bath for 3 hours. Water was added and the mixture extracted with ether (50 cc. 2 times). The extract was washed with 5% sulfuric acid, water, 10% sodium hydroxide and water. The residue was recrystallized from ethanol, m.p. 237–238° with decomposition. A sample was dried over P_2O_5 at 100° and 1 micron pressure for 1 hour and analyzed.

Analysis—$C_{21}H_{33}O_3N$	Calculated	C 72.16, H 10.10, N 4.01
	Found	C 72.77, H 9.88, N 4.70

Oxidation of the isolated acetate, m.p. 186–187°, with chromic acid yielded an acid, m.p. 186–187°, which depressed the melting point of the acetate. 2 oxidations were carried out. In the first, 10 mg. of the acetate were dissolved

in 0.4 cc. glacial acetic acid, and 0.5 cc. glacial acetic acid containing 10 mg. chromic acid was added and allowed to stand at room temperature for 16 hours. The excess chromic acid was reduced with methanol and the methanol evaporated. 10 cc. water were added and the mixture extracted with 100 cc. ether. The extract was washed with 5% sulfuric acid (10 cc.), water, 10% sodium hydroxide (10 cc. 2 times) and water. Evaporation of the ether yielded no neutral fraction. The alkaline washings were acidified, extracted with ether and washed with water. Evaporation of the ether yielded a residue of 11.2 mg. After recrystallization, the melting point was 185–186.5°. In the second oxidation, 25 mg. of the acetate were treated by the same method yielding crystals, m.p. 186–187°. A sample was dried over P_2O_5 at 100° and 1 micron pressure for 1 hour and analyzed.

Analysis—$C_{21}H_{34}O_4$	Calculated	C 71.96, H 9.78
	Found	C 72.37, H 10.11

This analysis is calculated for the hydroxy acid. It is believed that the acetyl group was saponified during contact with the 10% sodium hydroxide used during extraction.

On the basis of these data, it is suggested that the isolated compound, m.p. 186–187°, is 17a-methyl-D-homoetiocholanediol-3(α),17a-one-17 acetate, a rearrangement isomer of pregnanediol-3(α),17-one-20 acetate, m.p. 199–202°, a small amount of which was obtained in the following chromatogram (Chromatogram 5). This would be a rearrangement similar to the one believed to occur by Hirschmann and Hirschmann (1947), who obtained the homo rearrangement isomer 17a-methyl-Δ^5-D-homoandrostenediol-3(β), 17a(β)-one-20, from a patient with adrenal cortical carcinoma. In both cases the rearrangement isomer occurred to a greater extent than the precursor.

Fractions 31–37 (511 mg.) from Chromatogram 4, were rechromatographed, yielding impure crystalline material (225 mg.) which was combined with the mother liquors of fractions 25–30, reacetylated (220 mg.) and rechromatographed (Chromatogram 5). The material was dissolved in 2 cc. benzene and put on a column of 6 gm. aluminum oxide.

CHROMATOGRAM 5 (Fractions 31–37 of Chromatogram 4)

Fraction	*Eluant* (40 cc./fraction)	*Compound*
1– 6	benzene	0
	benzene-ether 10%	0
7– 9	benzene-ether 10%	m.p. 187–189°, m.p. 199–202°
10–11	benzene-ether 10%	m.p. 240–242°
12–16	benzene ether 20–30%	mixture
17–18	benzene-ether 40–50%	m.p. 178–182.5°

Fractions 7–9 from Chromatogram 5 yielded more of the compound (acetate of homo isomer?), m.p. 187–189°; when mixed with a sample, m.p. 186–187°, from the previous fractions (7–30 from Chromatogram 4), the melting point was not depressed. From the mother liquors of this compound a small amount of a compound, m.p. 199–202°, was obtained; when mixed with an authentic sample of pregnanediol-3(α),17-one-20 acetate, m.p. 199–201°, kindly supplied by Dr. S. Lieberman, the melting point was 199–201°. This is believed to be the precursor of the homo rearrangement isomer (?), m.p. 186–189°, just described. Fractions 10–11 yielded androstanediol-3(α),11(β)-one-17 acetate, m.p. 241–242°; when mixed with an authentic sample, m.p. 241–242°, the melting point was 241–242°. (For further charac-

terization of this compound, see fractions 34–46 of Chromatogram 6 from Fraction D, where large amounts were obtained.) Fractions 17–18 yielded a compound, m.p. 178–182.5°, which depressed the melting point of androsterone.

Fraction Dα. Fraction D (4.4 gm. containing 2.68 gm. 17-ketosteroids) was precipitated with digitonin yielding a β fraction (0.015 gm., vide infra) and an α fraction (3.3 gm.). The α fraction was acetylated (3.8 gm.), dissolved in 100 cc. benzene-petroleum ether 4:1 and put on a column of 114 gm. aluminum oxide (Chromatogram 6).

CHROMATOGRAM 6 (Fraction Dα, acetylated)

Fraction	*Eluant* (100 cc./fraction)	*Compound*	*17-ketosteroid Value (mg.)*
1– 2	benzene-pet. ether 4:1	0	
3– 4	benzene-pet. ether 4:1	3-chloro-Δ⁵-androstenone-17	
5– 6	benzene-pet. ether 4:1	mixture (rechromatographed)	
7–16	benzene-pet. ether 4:1	androsterone acetate	
17–33	benzene	androsterone acetate	
	benzene-ether 5%	androsterone	
34–46	benzene-ether 25%	11-OH-androsterone acetate etiocholanol-3(α)-one-17 11-OH-androsterone	
47–52	benzene ether 50%	oil	308
53–61	benzene-ether 75%	oil	108
62–67	ether-ethanol 1%	oil	144
68–73	ether-ethanol 5–10%	oil	306
74–85	ether-ethanol 25–75%	m.p. 235–236.5°	

Fractions 3–4 yielded 18 mg. 3-chloro-Δ⁵-androstenone-17, m.p. 153.5–156°; when mixed with an authentic sample, m.p. 155–156°, the melting point was 153.5–156°. The brownish mother liquors were evaporated, taken up in 50 cc. ether, washed with 10% sodium hydroxide and water, the ether evaporated, the residue dissolved in 4 cc. benzene-petroleum ether 1:1 and put on a column of 5 gm. aluminum oxide. Elution with the solvent yielded 12.6 mg. crystals, m.p. 153.5–156°, which did not depress the melting point of 3-chloro-Δ⁵-androstenone-17.

Fractions 5–6 yielded 273 mg. of a mixture of crystals. This was rechromatographed, yielding androsterone acetate, m.p. 158.5–160°; when mixed with an authentic sample, m.p. 158–161.5°, the melting point was 158.5–160°. Crystals melting over a wide range were also obtained. These were saponified and rechromatographed. Free androsterone, m.p. 175–179°, was obtained; when mixed with an authentic sample, m.p. 180–182°, the melting point was 179.5–181.5°. The mother liquors were rechromatographed (Chromatogram 7). The material (121 mg.) was dissolved in 5 cc. benzene-petroleum ether 3:2 and put on a column of 4 gm. aluminum oxide.

CHROMATOGRAM 7 (Mother liquors of fractions 7–16 from Chromatogram 6)

Fraction	*Eluant* (10 cc./fraction)	*M.P.*
1– 2	pet. ether	0
3–17	benzene-pet. ether 1:9–1:1	160–164.5°
18	benzene	144–154°
19	benzene	132–134°
20	benzene-ether 3:1	177–182°
21–22	benzene-ether	oil
23–26	ether, ethanol	0

Fractions 3–17 yielded androsterone acetate, m.p. 160–164.5°; when mixed with an authentic specimen, the melting point was not depressed. Fraction 19 yielded a small amount of a compound, m.p. 132–134°, which depressed the melting point of androstanedione-3,17, m.p. 132–134°. Free androsterone, m.p. 177–182°, was obtained from fraction 20; when mixed with an authentic sample, m.p. 182–183°, the melting point was 181–183°.

Fractions 17–33 from Chromatogram 6. This material (266 mg.) was a brownish-yellow gum and was rechromatographed, yielding oils and a small amount of free androsterone. These oils were reacetylated (172 mg.) and rechromatographed. Only oils were eluted, whose total 17-ketosteroid value was 138 mg.

Fractions 34–46. 640 mg. androstanediol-3(α),11(β)-one-17 acetate (11-OH-androsterone acetate), m.p. 237–242°, was obtained from this fraction, with additional material in the mother liquors. The isolation and identification of this compound from this fraction has been reported previously (Miller *et al.*, 1945). The compound was dehydrated in 2 ways, to determine if the product were identical with the Δ?-androstenol-3(α)-one-17 isolated from the acid hydrolyzed urine of this patient (Dorfman *et al.*, 1945). The dehydrated product was oxidized to the diketone and compared with Δ^9-androstenedione-3,17, to determine if the double bond were at C_9.

(a) *Acid Dehydration.* The dehydration product of androstanediol-3(α),11(β)-one-17 acetate obtained by acid dehydration has been reported previously (Miller *et al.*, 1945) to yield a product, m.p. 188–191°; when this compound was mixed with the compound, m.p. 186–189°, obtained from the acid hydrolyzed urine of this patient (Chromatogram 10), there was no depression of melting point.

(b) *$POCl_3$ Dehydration.* 15 mg. androstanediol-3(α),11(β)-one-17 acetate, m.p. 238–239°, were dissolved in 0.04 cc. pyridine containing 0.01 cc. $POCl_3$ and allowed to stand at room temperature for 24 hours. Water was added, the mixture extracted with 100 cc. ether and washed with 5% sulfuric acid, sodium carbonate and water. 13.6 mg. of brownish crystals were obtained. 3 crystallizations from absolute methanol yielded crystals, m.p. 188.5–190°. The mother liquors were chromatographed on 400 mg. aluminum oxide in 2 cc. benzene-petroleum ether 1:1. Fraction 1, eluted with 50 cc. of the solvent, yielded crystals, m.p. 188.5–190°, which did not depress the melting point of the product, m.p. 188–191°, obtained by acid dehydration. Fraction 2 yielded starting material, m.p. 236.5–238°.

(c) *Chromic Acid Oxidation.* About 10 mg. $\Delta^{9 \text{ or } 11}$-androstenol-3($\alpha$)-one-17 acetate (obtained by acid dehydration of androstanediol-3(α),11(β)-one-17 acetate), slightly impure, were saponified yielding crystals, m.p. 184–185°, which were dissolved in 0.3 cc. glacial acetic acid containing 3 mg. chromic acid and allowed to stand 3 hours at 28°. Water was added, the mixture extracted with 100 cc. ether and washed with 5% sulfuric acid, sodium carbonate and water. 6.2 mg. were obtained and chromatographed on 200 mg. aluminum oxide in 5 cc. benzene-petroleum ether 1:4. Fractions 2–6, each eluted with 10 cc. of the solvent, were combined and after crystallization from aqueous methanol, melted at 150–162°. Fractions 7–8, each eluted with 10 cc. benzene-petroleum ether 1:1, were combined and after crystallization from aqueous acetone melted at 150–152°. When this compound was mixed with Δ^9-androstenedione-3,17, m.p. 150–152°, kindly

supplied by Dr. C. W. Shoppee, the melting point was 149.5–152°.

The mother liquors of fractions 34–46 (1.120 gm.) from Chromatogram 6 were evaporated, dissolved in 45 cc. benzene and put on a column of 20 gm. aluminum oxide (Chromatogram 8).

CHROMATOGRAM 8 (Mother liquors of fractions 34–46 from Chromatogram 6)

Fraction	*Eluant* (100 cc./fraction)	*Compound*
1– 7	benzene	11-OH-androsterone acetate
8–11	benzene-ether 5–10%	11-OH-androsterone acetate
12–16	benzene-ether 25–50%	etiocholanol-3(α)-one-17
17–18	benzene-ether 75%, ether	0
19–21	ether-ethanol 1%	11-OH-androsterone
22	ether-ethanol 5–10%	trace
23	ether-ethanol 25–75%	0

Fractions 1–11 from Chromatogram 8 yielded 246 mg. androstanediol-3(α),11(β)-one-17 acetate, m.p. 238–241°; when mixed with an authentic sample, the melting point was not depressed. Fractions 12–16 yielded 14.6 mg. etiocholanol-3(α)-one-17, m.p. 139–140° and 148.5–150°; when mixed with an authentic sample, the melting point was not depressed. Fractions 19–21 yielded crystals, m.p. 193.5–196°; when mixed with androstanediol-3(α),11(β)-one-17, m.p. 194–196.5°, the melting point was 193.5–196°. The mother liquors from fractions 1–11 were evaporated (169 mg.) and combined with the following fractions 47–73.

Fractions 47–73 from Chromatogram 7. The total 17-ketosteroid value of this oily material was 862 mg.

Fractions 74–85. This dark brown semicrystalline material was taken up in ether, washed with 10% sodium hydroxide until the aqueous phase was colorless, and with water, and recrystallized from acetone, yielding crystals, m.p. 235–236.5°. When mixed with crystals, m.p. 235–237°, obtained from Fraction C (Chromatogram 4), the melting point was not depressed. The compound has not been identified.

Fraction Dβ. This fraction (105 mg.) was acetylated (103 mg.), dissolved in 8 cc. benzene-petroleum ether 1:3 and put on a column of 3 gm. aluminum oxide (Chromatogram 9).

CHROMATOGRAM 9 (Fraction Dβ, acetylated)

Fraction	*Eluant* (40 cc./fraction)	*M.P.*
1– 2	benzene-pet. ether 1:3	trace
3– 5	benzene-pet. ether 1:3	152–162°
6– 8	benzene-pet. ether 1:3	trace
9–14	benzene-pet. ether 1:1	mixture
	benzene-pet. ether 3:1	trace
	benzene	trace
15–16	benzene-ether 3:1	219–222°
17–25	ether, ethanol	trace

Fractions 3–5 yielded 40 mg. crystals which after 3 crystallizations melted at 152–162°; when mixed with dehydroisoandrosterone acetate, m.p. 163–165°, the melting point was 160–163.5°.

Fractions 15–16 yielded crystals, m.p. 217–221°, which were rechromatographed (11.8 mg.), yielding 2–3 mg. of a compound, m.p. 219–222°, which appeared to have a 17-keto group on the basis of the Zimmermann reaction.

HYDROLYZED URINE

This urine represents the residual urine after benzene extraction of the unhydrolyzed urine (95.1 liters). It was acidified with 15% concentrated hydrochloric acid (by volume) and refluxed for 15 minutes. Approximately one half (5.3 gm.) of the total neutral fraction (10.3 gm. containing 3.38 gm. 17-ketosteroids) was fractionated as shown in Fig. 2.

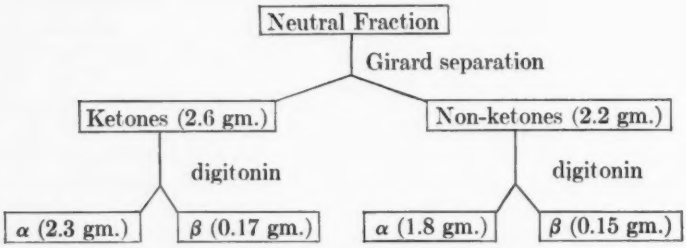

FIG. 2. Fractionation of Hydrolyzed Urine.

KETONIC FRACTION

The ketonic α fraction was investigated. The chromatogram of the free (non-acetylated) material yielded only oils. The eluates were combined, acetylated (2.3 gm.), dissolved in 30 cc. benzene-petroleum ether 1:1 and put on a column of 50 gm. aluminum oxide (Chromatogram 10).

CHROMATOGRAM 10 (Ketonic α fraction, acetylated)

Fraction	*Eluant* (50 cc./fraction)	*Compound*	*Wt. (gm.)*
1– 6	benzene-pet. ether 1:3	0	
7	benzene-pet. ether 1:2	trace	
8– 9	benzene-pet. ether 1:2	m.p. 119–123°	
10–11	benzene-pet. ether 1:2	oil	
12–14	benzene-pet. ether 1:2	3-chloro-Δ^5-androstenone-17	
15	benzene-pet. ether 1:1	oil	
16–27	benzene-pet. ether 1:1	Δ^9-androstenolone acetate	
	2:1	Δ^9-androstenolone acetate	
	3:1	Δ^9-androstenolone acetate	
28	benzene-pet. ether 3:1	oil	
29–37	benzene	oil	0.5
38–47	benzene-ether 5–20%	oil	0.2
48–59	benzene-ether 25%	11-OH-androsterone acetate	
60–70	benzene-ether 30–90%	oil	0.2
71–78	ether-ethanol 1– 5%	oil	0.26
79–85	ether-ethanol 10–75%	oil	0.22

Fractions 8–9 yielded a mixture of crystals. These were combined (25.2 mg.) and rechromatographed, yielding 2–3 mg. crystals, m.p. 119–123°, which gave a strongly positive Zimmermann test, negative Beilstein test, and reduced bromine.

Fractions 12–14 yielded 7.6 mg. 3-chloro-Δ^5-androstenone-17, m.p. 149.5–153°; when mixed with an authentic sample, m.p. 155–157°, the melting point was 152.5–155°. Recrystallization raised the melting point to 153–156°.

Fractions 16–27 yielded 49.0 mg. Δ^9-androstenol-3(α)-one-17 acetate, m.p. 186–189°; when mixed with an authentic sample of Δ^9-androstenol-

3(α)-one-17 acetate obtained by acid dehydration of androstanediol-3(α),11(β)-one-17 acetate, the melting point was not depressed.

Fractions 29–37 (0.5 gm.) which might be expected to contain androsterone acetate, were combined with the mother liquors from the previous fractions 16–28, as well as with all the mother liquors containing androsterone acetate from the unhydrolyzed urine (Fractions C, D), saponified and rechromatographed. The combined fractions (1 gm.) were dissolved in carbon tetrachloride and put on a column of 25 gm. aluminum oxide. 62 mg. androsterone, m.p. 176–179°, were obtained; when mixed with an authentic sample, m.p. 180–181°, the melting point was 178–180°. 66 mg. etiocholanol-3(α)-one-17, m.p. 137–138° and 146–147°, were obtained; when mixed with an authentic sample, m.p. 137–138° and 147–148°, the melting point was 136–137° and 147–148°.

Fractions 48–59 (0.3 gm.) were rechromatographed, yielding 50.7 mg. androstanediol-3(α),11(β)-one-17 acetate, m.p. 240–242°; when mixed with an authentic sample, m.p. 240–242°, the melting point was not depressed.

NON-KETONIC FRACTION

The non-ketonic β fraction from a 25.4 liter sample of hydrolyzed urine from this patient was investigated. The isolation of Δ^5-pregnenediol-3(β), 20(α) in small amount (0.18 mg./liter) as the diacetate and monoacetate has been reported previously (Schiller *et al.*, 1945) and is mentioned here for completeness. 2 unidentified compounds were also isolated from this fraction: a compound, m.p. 96.5–98°, eluted with benzene-petroleum ether 1:9 which upon saponification yielded a product, m.p. 131.5–132.5°; and a compound, m.p. 166.5–167°, eluted with benzene-petroleum ether 1:4 which was not allopregnanediol-3(β),20(α) diacetate.

SUMMARY

The following 13 steroid metabolites (8 ketones and 5 non-ketones) were isolated from urine of a patient with (probable) adrenal cortical hyperplasia, in order of decreasing amounts: androstanediol-3(α), 11(β)-one-17; pregnanetriol-3(α),17,20; pregnandiol-3(α),20(α); (?) 17a-methyl-D-homoetiocholanediol-3(α),17a-one-17; androsterone; etiocholanol-3(α)-one-17; Δ^9-androstenol-3(α)-one-17; 3-chloro-Δ^5-androstenone-17; Δ^5-pregnenediol-3(β),20(α); dehydroisoandrosterone; etiocholanediol-3(α),17(α); allopregnanediol-3(α),20(α) and pregnanediol-3(α),17-one-20. The amounts isolated are reported.

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ASCORBIC ACID CONTENT OF ADRENAL GLANDS OF YOUNG ALBINO RATS AFTER COLD AND OTHER STRESSES[1]

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INFANT albino rats are unable to regulate their body temperatures under cold stress prior to 18 days of age. This was first reported by Hill (1947) and was corroborated by Fairfield (1948), Adolph (1948) and Holtkamp *et al.* (1949). The possibility that the adrenals of infant rats are incapable of active response to ACTH is suggested by the investigations of Emery and Winter (1934) while the report of Holtkamp *et al.* leads to speculation that, whereas the adrenals of the infant rat may be reactive to stimulation, their products (corticosteroids) may still not be utilized by the rat to maintain its temperature under cold stress. In the light of this possibility it was deemed pertinent to determine the ability of infant rats' adrenals to respond to the stimulation of other types of stress than cold.

METHODS AND MATERIALS

Continuous temperature records were obtained by means of thermopiles inserted through the anus and rectum into the colon and connected to a Brown electronic recording potentiometer as previously described (Buchanan and Hill, 1947). The thermopiles were contained within ureteral catheters with outside diameters of 2.4 mm. After insertion of a thermopile it was securely fastened to the tail of the rat with adhesive tape and both thermopile and tail were led out of a small, well-ventilated can within which the animal was confined during the stress period; the arrangement was such as to prevent the rat from pulling out or biting through the thermopile.

Litters were selected from a mixed, inbred strain of Wistar albino rats. The chief criterion used was that the litter must be large enough to provide at least one animal to be used as a control after the experimental animals had been selected at random.

The total number of rats used was 211 of which 115 were 13 days of age. Thirty-seven of the 13-day-old rats served as controls, 19 were cold-stressed

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at 5° ± 1°C. for 3 hours, 17 were subjected to cold stress for 1 hour in combination with indwelling thermopiles, 6 were heat-stressed for 1 hour at 37° ± 0.3°C., 18 had thermopiles inserted and kept in situ for 1 hour at "normal" or average room temperature of 28° to 30.5°C., 6 were subjected to heat stress for 1 hour in combination with indwelling thermopiles, 6 had combined heat and ergotoxine stress for 1 hour and, finally, 6 were stressed for 1 hour with heat and ergotoxine combined with indwelling thermopiles.

Seventy-five 32-day-old rats were used; of these 30 served as controls and the others were divided into 3 groups of 15 each. One group was subjected to 3-hour cold stress, another was cold-stressed for 1 hour with indwelling thermopiles and the third group had thermopiles in situ in their colons for 1 hour at "normal" room temperature.

Twenty-one 50-day-old rats were divided into 3 groups of 7 each. One group was used for controls, the second had thermopiles implanted for 1 hour and the third was cold-stressed for 1 hour with indwelling thermopiles.

Ergotoxine-stressed rats received intraperitoneal injections of 4.5 mg./kg. body weight of ergotoxine ethanesulfonate[2] dissolved in 6.25% ethyl alcohol as described by Buchanan *et al.* (1948). Environmental temperature ranges for the 32- and 50-day-old rats were the same as specified for 13-day-old animals. The rats were not fasted prior to being stressed.

At the end of the stress periods the rats were decapitated and bled. The adrenals were removed through a dorsal approach and left beneath the skin to prevent drying while those of the other animals in a given litter were being removed. Each adrenal was then weighed on a torsion type microbalance, dropped into a tube containing 1 ml. of 4% trichloroacetic acid and placed in a deep-freeze unit at −10°C. When subsequently removed from the deep-freeze unit the adrenals were homogenized and analyzed chemically for their ascorbic acid contents. It has been considered justifiable, in the light of Sayers and Sayers (1947) having shown the decrease in adrenal ascorbic acid after stress periods of 1 hour or less to be an indication of adrenal activity, to evaluate adrenal activity in our stressed animals on the basis of reductions in adrenal ascorbic acid to levels below those in litter-mate controls.

The chemical analyses of the ascorbic acid content of the adrenals were made by the Roe and Kuether 2,4-dinitrophenylhydrazine method (1943). A Leitz photoelectric colorimeter was used with a filter having a maximum transmission at 525 millimicrons. Results on left adrenals from control rats were well within the permissible range of ±5% error when compared with the values obtained from the right adrenals of the same animals. The percentage-transmission reading was converted to gamma/100 mg. of adrenal tissue and so recorded in the protocols.

Hyper- and hypothermic responses to individual and combined stresses, in terms of degree-minutes (Buchanan, Roberts, and Robinson, 1948) were measured with a planimeter.

RESULTS

Body Temperature Reactions to Stress. In rats kept in the "normal" temperature environment (28° to 30.5° C.) minimal hypothermic reactions were associated with thermopiles implanted in their colons

[2] Kindly furnished by the Wellcome Research Laboratories, Tuckahoe, New York.

(Fig. 1); these were least marked in the 50-day-old animals and of greatest magnitude in the 13-day-old group. The hypothermias in the former group averaged 18.7 and in the latter 78.9 degree-minutes during a 1-hour period. In the 32-day-old rats hypothermias from the thermopile alone averaged 31.3 degree-minutes.

Cold stress, when combined with indwelling thermopiles, accounted for a hypothermic reaction of 552.2 degree-minutes in 1 hour

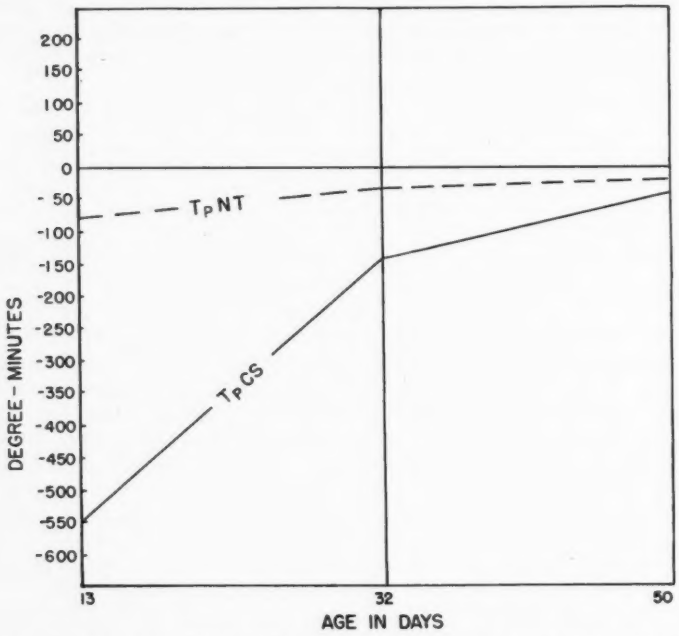

FIG. 1. Total hypothermic reactions of 13-, 32- and 50-day-old rats during 1 hour of thermopile stress at room temperature (28° to 30.5°C.) and during 1 hour of thermopile-cold stress (5° ± 1°C.). TpNT indicates thermopile stress at "normal" room temperature and TpCS indicates thermopile-cold stress.

in the 13-day-old rats, 141.0 degree-minutes in 32-day-old animals and only 36.7 degree-minutes in the 50-day group (Fig. 1).

Hyperthermia, amounting to 166.4 degree-minutes in 1 hour, resulted from combined thermopile and heat stress in the 13-day-old rats and this was increased to 191.2 degree-minutes when administration of ergotoxine was superimposed upon these 2 stresses (Fig. 2).

Degree-minutes of hypothermia could not be determined for the 13- and 32-day-old rats which were cold-stressed for 3 hours because their body temperatures, after 1½ to 2 hours, had descended to levels below the range of the electronic potentiometer or of any thermometers available.

Adrenal Ascorbic Acid. Ascorbic acid in gamma/100 mg of adrenal

TABLE 1. ASCORBIC ACID IN GAMMA/100 MG. ADRENAL TISSUE AND STATISTICAL ANALYSIS OF DIFFERENCES BETWEEN EXPERIMENTAL AND CONTROL RATS

Age in days	Type of stress	Mean ascorbic acid content	Range	Number of animals	Probability
13	None (Control)	240.8	437.0–137.0	37	
13	CS 3-hour	230.1	416.5–146.7	19	>0.5
13	TpCS 1-hour	204.1	437.0–135.0	17	>0.5
13	H 1-hour	215.1	263.0–158.5	6	0.27
13	TpNT 1-hour	184.7	333.0–100.0	18	<0.01
13	TpH 1-hour	156.8	187.3–117.0	6	<0.01
13	HE 1-hour	155.4	184.3–108.0	6	<0.01
13	TpHE 1-hour	147.4	240.0– 96.5	6	<0.01
32	None (Control)	250.9	367.0–111.3	30	
32	CS 3-hour	190.1	391.0–106.2	15	<0.01
32	TpCS 1-hour	174.8	303.0– 92.0	15	<0.01
32	TpNT 1-hour	162.0	220.0– 75.0	15	<0.01
50	None (Control)	303.0	375.0–238.0	7	
50	TpNT 1-hour	225.8	299.4–175.5	7	0.08
50	TpCS 1-hour	217.9	288.0–168.0	7	0.05

LEGEND: CS, cold stress; H, heat; HE, heat and ergotoxine; TpCS, thermopile and cold stress; TpH, thermopile and heat; TpHE, thermopile, heat and ergotoxine; TpNT, thermopile at "normal temperature."

tissue, as determined for control and experimental 13-day-old rats, is shown in the first part of Table 1. The 3-hour cold stress group showed a depletion of only 10.7 gamma (4%) under the mean level of the control animals. One hour of cold stress combined with indwelling thermopiles resulted in an average reduction in ascorbic acid of 15.2% which was considerably more than that due to 3-hour cold stress without thermopiles but which was not sufficiently great to be statistically significant (Table 1). Heat stress, continued for 1 hour, accounted for an average depletion in ascorbic acid of 10.7% which also lacked statistical significance. The other 4 stresses applied to the

13-day-old rats (thermopile, thermopile-heat, heat-ergotoxine and thermopile-heat-ergotoxine) all produced statistically significant depletions; the least significant of these was that resulting from the thermopile at "normal" room temperature.

All 3 experimental groups of 32-day-old rats, respectively subjected to 3-hour cold stress, 1-hour thermopile-cold stress and thermopile at "normal" room temperature, had depletions of adrenal

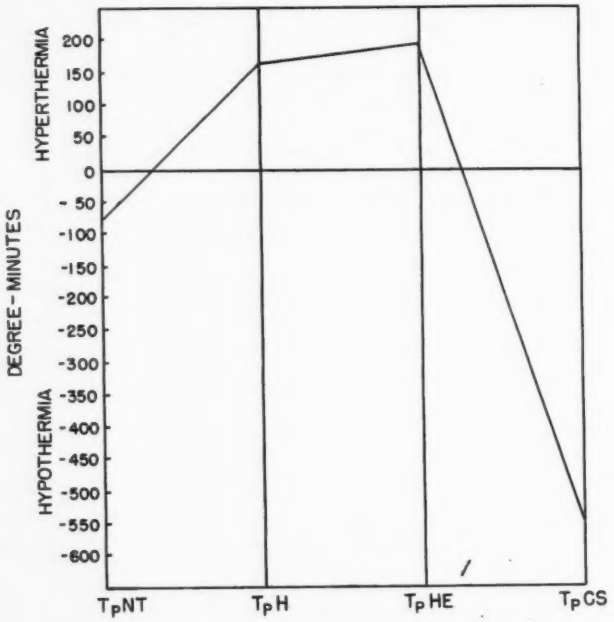

FIG. 2. Comparison of the total thermal reactions of 13-day-old rats during 1 hour of thermopile stress (TpNT), thermopile heat stress (TpH), thermopile-heat-ergotoxine stress (TpHE) and thermopile-cold stress (TpCS).

ascorbic acid which were of considerable magnitude and were all statistically significant when compared with 32-day-old control values (Table 1).

Ascorbic acid concentrations in 50-day-old rats' adrenals were not significantly reduced, when compared with control values, by 1 hour of thermopile or thermopile-cold stress (Table 1). Thermopile-cold stress did, however, account for a mean depletion of 85.4 gamma (24.8%) which was very close to statistical significance.

Statistical evaluation[3] of our results was accomplished through

[3] The authors wish to express their appreciation to Dr. H. J. Dodge, Associate Professor of Public Health and Preventive Medicine, for invaluable assistance in setting up the statistical analysis of the data presented herein.

the use of the conventional method of calculation of the standard error of the difference between 2 means. An experimental group which, when compared with appropriate controls, gave a probability figure of 0.01 or less was considered statistically significant (Table 1). In order to eliminate discrepancies due to inter-litter differences in ascorbic acid levels a second method,—Student's "t" test for analysis of paired differences,—which was especially designed for small sampling (Freeman, 1947), was applied to the data obtained from single litters; this gave more significant figures than those obtained by the first method and presented in Table 1. When Student's "t" test was applied to the data from 50-day-old rats the probability figure for thermopile stress at room temperature was decreased from 0.08 to approximately 0.03 and for thermopile-cold stress from 0.05 to approximately 0.025. It seems certain, therefore, that statistical significance has been credited to the data presented herein (Table 1) only where it is entirely justifiable.

DISCUSSION

The mild hypothermias associated with indwelling thermopiles may be partly, but certainly not entirely, due to the thermopiles. Young rats, when isolated from their fellows and kept in an environmental temperature of 28° to 30.5° C. (our "normal" room temperature) tend to lose heat to the environment with resultant decrease in body temperature of about the same degree (determined with thermometers) as observed in those with indwelling thermopiles. This tendency normally becomes less marked as the rats approach the age of optimum thermoregulatory ability (60 to 300 days according to Hill, 1947) and the same was certainly true of those observed with thermopiles fixed in place in their colons (Fig. 1).

The rather wide range in adrenal ascorbic acid levels observed in both control and experimental groups of rats (Table 1) cannot be accounted for positively but may have been partly due to the lack of thermostatic control of the temperature of the room in which the stock animals were housed. Because the interval between the carrying out of the experimental procedures on the first and last litters in a given series was sometimes as long as 60 days, seasonal variations in the outdoor temperature often accounted for wide fluctuations in the temperature of the animal room. In spite of inter-litter variability there was a definite trend toward higher concentrations of ascorbic acid in the adrenals of 50-day-old control rats than in those of either of the younger groups (Fig. 3).

The values for adrenal ascorbic acid in 13- and 32-day-old rats after thermopile stress at "normal" room temperature are particularly striking when compared graphically with the rising values in control animals of the same ages (Fig. 3). When cold stress was combined with the thermopile ascorbic acid depletion was less than that pro-

duced by the thermopile alone in 13- and 32-day-old rats and slightly greater in 50-day-old animals. The failure of the combined stress to account for as low a level of ascorbic acid in the 2 younger groups as was produced by the thermopile alone may have been because their hypothermic reactions, as shown in Figure 1, were of sufficient magnitude to partially block the enzymatic reactions responsible for the depletion from the thermopile at room temperature. The likelihood

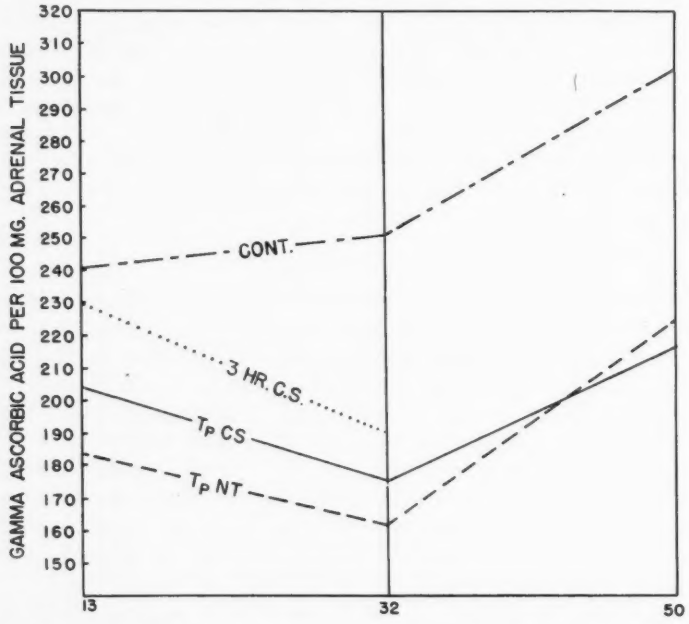

FIG. 3. Ascorbic acid content in gamma/100 mg. adrenal tissue after thermopile stress at room temperature (TpNT), after thermopile-cold stress (TpCS), both for 1 hour and after 3-hour simple cold stress (CS) compared with the levels in control animals.

that enzymatic activity is responsible for the ascorbic acid depletion from the thermopile alone and that cold stress partially blocks this activity appears more reasonable in the light of the observations in 13- and 32-day-old rats that less depletion resulted from 3-hour cold stress without thermopile than from either 1-hour thermopile or thermopile-cold stress (Fig. 3). It is also of interest, in this connection, that 1-hour cold stress with thermopile at 50 days of age, when hypothermia was minimal (Fig. 1), accounted for slightly more ascorbic acid depletion than the thermopile at room temperature.

Increased activity of the adrenal glands in response to 3-hour cold stress in 32-day-old rats as compared with that in 13-day animals, on the basis of ascorbic acid depletion (Fig. 3), correlates well with

the increasing ability of rats between these ages to maintain their body temperatures reasonably well.

The relative failure of the adrenals of 13-day-old rats to react to cold stress contrasts strongly with the effect of heat stress in the same age-group (Table 1). Combined thermopile and heat stress (TpH, Table 1) calls forth nearly maximum depletion of ascorbic acid as becomes evident when ergotoxine stress is superimposed upon this combination (TpHE, Table 1); the addition of this potent stress elicits but very little additional depletion. That ergotoxine, per se, is a severe stress is evident when the depletion due to heat and ergotoxine (HE) is compared with that due to heat stress alone (H, Table 1).

It must be noted, with respect to multiple stresses, that conflicting results are obtained for a single stress when one attempts to extract its value from the results obtained with multiple stresses. That one cannot obtain the true depletion value of thermopile stress by subtracting the value of another stress from the results obtained by combining it with the thermopile is readily apparent upon examination of Table 1. The statement may therefore be made, on the basis of the data reported, that the effects of singly applied stresses are not numerically additive. This was to be expected since Long (1947) has shown that the depletion of adrenal ascorbic acid in response to repeated injections of ACTH reaches a maximum in about 3 hours. Sayers *et al.* (1948) corroborated Long's findings and reported further that the concentration of ascorbic acid in the adrenals is depleted by injected ACTH to a level which may be regarded as constant and that this depletion is of a logarithmic nature, and so constantly so, that they have used it as a means of assaying ACTH. They also found that the adrenals, in response to stress, can be depleted of ascorbic acid to a certain level beyond which no additional stress will produce further significant depletion.

The "general alarm reaction" described by Selye (1947) is apparently elicited by the thermopile as used in these and other experiments (Buchanan and Hill, 1947; Buchanan *et al.*, 1948). This is indicated by statistically significant adrenal ascorbic acid depletion in both 13- and 32-day-old rats and a depletion approaching statistical significance in 50-day-old animals from 1 hour of simple thermopile stress. The failure of 13-day-old rats to maintain their body temperatures when placed in a cold environment is therefore of especial interest since it occurs in spite of an apparent increase in adrenal activity produced by indwelling thermopiles.

Depletion of adrenal ascorbic acid of significant degree in 32-day-old rats subjected to simple cold stress (Fig. 3), associated with marked reduction in magnitude of their hypothermic reactions (Fig. 1), quite definitely indicates adrenal participation in thermoregulation at this age level; this is in agreement with the observations

of Holtkamp *et al.* (1949) and, together with the evidence of thermopile-induced adrenal activity in 13-day-old animals which is ineffective against cold, suggests that stimulation of cold-receptor mechanisms is responsible, through humoral or nervous mediation, for a particular type of adrenal activation. Such cold-receptor mechanisms might include not only those which are peripherally situated but also those in the hypothalamus which are apparently affected by decreases in the temperature of the blood flowing through the area (Geiger, 1932). Immaturity of the hypothalamic centers responsible for thermoregulation may, on the basis of previous work (Buchanan and Hill, 1947; Buchanan and Roberts, 1948), represent an important gap in the chain of events necessary for cold stimulation to elicit an effective adrenal response. The possibility must be considered seriously that immaturity of the thermoregulatory centers in the hypothalamus accounts for a failure of peripheral vasoconstriction and other heat-conservation reactions to cold stress, to be rapid enough or of sufficient magnitude to maintain body temperature at such levels as are essential for the enzymatic activity which would otherwise contribute to the production of heat.

SUMMARY

Significant depletions of adrenal ascorbic acid were not obtained in 13-day-old albino rats subjected to either simple 3-hour cold stress or 1-hour thermopile-cold stress. The latter stress accounted, however, for an average hypothermic reaction of 555.2 degree-minutes in 1 hour. Adrenal ascorbic acid was significantly depleted by both 3-hour cold stress and 1-hour thermopile-cold stress in 32-day-old rats whose hypothermic reactions to the latter stress averaged only 141.0 degree-minutes. The adrenals of 50-day-old rats were not significantly depleted when subjected to thermopile-cold stress for 1 hour. The very near approach to statistical significance in this group, however, makes it likely that a moderate increase in the severity of the cold stress (a longer period of stress or a temperature lower than $5° \pm 1°$ C.) might have resulted in significant depletions.

Significant depletions of ascorbic acid in the adrenals of 13-day-old rats obtained by thermopile stress at "normal" room temperature (28° to 30.5° C.), thermopile-heat stress, heat-ergotoxine stress and thermopile-heat-ergotoxine stress appear to justify the conclusion that the adrenals of non-thermoregulatory rats are capable of participation in the "general alarm reaction" of Selye. The failure of cold stress to produce either an adequate thermoregulatory reaction or significant depletion of adrenal ascorbic acid in 13-day old albino rats must therefore be due to immaturity of some part of the thermoregulatory mechanism between the cold-receptor organs and the adrenals and/or to depression of body temperature below the level essential to the enzymatic reactions involved in the production of corticos-

teroids. On the basis of previous investigations it is probable that immaturity of the hypothalamus is one of the factors involved and that body temperature descends to a level too low for the enzymatic reactions that contribute to maintenance of body temperature, because of failure of adequate heat-conservation reactions to cold stress.[4]

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[4] An article by Joseph W. Jailer on "The Pituitary-Adrenal Relationship in the Infant Rat" appeared in the Proceedings of the Society for Experimental Biology and Medicine 72: 638, (Dec.) 1949, after our report had been prepared for publication. The conclusions of Jailer agree with and tend to support the more extensive observations reported herein.

ACTIVATION OF THE ADENOHYPOPHYSIS BY INTRAVENOUS INJECTIONS OF EPINEPHRINE IN THE ATROPINIZED RABBIT

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INTRODUCTION

MARKEE, Sawyer and Hollinshead (1948) stimulated the release of luteinizing hormone (or total gonadotrophin) from the rabbit hypophysis by the intrahypophyseal injection of small amounts of epinephrine (Adrenalin). After receiving 4 injections of 0.05 ml. epinephrine (1 mg./ml.), 5 out of 10 rabbits ovulated. However, intravenous and intracarotid injections of much higher dosages, up to the MLD, led to completely negative results (*op. cit.*, and Marshall and Verney, 1936). These findings suggested that a discrete adrenergic mechanism, localized in the region of the hypophysis, might control pituitary activation. The presence of such an adrenergic mechanism in the reflex by which coitus stimulates LH release in the rabbit was affirmed by blocking copulation-induced ovulation with the adrenergic blocking agent Dibenamine (Sawyer, Markee and Hollinshead, 1947). Subsequent work revealed that the pituitary activation reflex also contains an atropine-blocked component which apparently precedes the Dibenamine-blocked mechanism (Sawyer, Markee and Townsend, 1949). The release of ovulating hormone in the rat was also found to be blocked by the appropriately timed injection of either atropine or Dibenamine (Sawyer, Everett and Markee, 1949; Everett, Sawyer, and Markee, 1949).

A more recent investigation (Sawyer, Markee and Everett, 1950) deals with the mechanism by which adrenolytic agents block activation of the hypophysis. It was found that adrenergic blocking agents which prevent LH release will protect against the toxic effects of a twice-lethal dose of epinephrine within a few seconds after injection of the blocking agent. The present study was initiated merely to test in a parallel manner the mechanism of atropine-block. The results reveal that the dosage of atropine which blocks copulation-induced ovulation develops practically immediate protection against a twice-lethal dose of acetylcholine and eserine. However, we found to our surprise that atropine also protects against a twice-lethal dose of epinephrine.

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This phenomenon of atropine-protection against lethal doses of epinephrine has also been reported by Auer and Gates (1917), Bariety and Kohler (1947) and Stone and Loew (1949). Atropine prevents the lethal pulmonary edema by counteracting certain isolated physiological effects of epinephrine (e.g., peripheral vasoconstriction) rather than by virtue of specific adrenolytic properties. However, the question arises as to whether atropine blocks LH release by counteracting adrenergic function. If not, the atropinized rabbit provides an excellent research animal in which to test the effects of high systemic dosages of epinephrine on the pituitary-activation mechanism. The present paper reports that, under these conditions, LH release has been stimulated by intravenous injections of epinephrine.

MATERIALS AND METHODS

Certain data, to be indicated by footnotes to the tables, have been taken from earlier publications (Sawyer *et al.*, 1949, 1950), and cumulative results are presented here. The results also include new data from 91 rabbits. The latter include 32 immature males and females averaging 2.0 kg. in weight and employed only in tests of survival following injections of the various drugs. Also included are 59 mature females ranging in weight from 2.4 to 5.0 kg. (average 3.2 kg.), used both in survival tests and experiments on the stimulation of LH-release from the hypophysis.

Most of the routine procedures, including the methods of preparing and administering drugs, have been described previously (Sawyer, Markee and Townsend, 1949; Sawyer, Markee and Everett, 1950). Characteristically atropine sulfate (30 mg./kg.) was injected into one marginal ear vein within 15 seconds and a twice-lethal dose of epinephrine (1 mg./kg.) or acetylcholine-eserine (450–225 μg./kg.) into the other ear within the next 45 seconds, the whole injection being completed within one minute. The last solution was prepared by mixing a solution of acetylcholinec hloride (0.9 mg./ml. in unbuffered Ringer-Locke) with eserine sulfate (0.45 mg./kg.) in equal parts, the final concentrations being 450 μg./ml. and 225 μg./ml., respectively. The estrogen which was employed in the second series of experiments to facilitate the LH-release mechanism (cf. Dury and Bradbury, 1943; Sawyer, 1949; Sawyer and Markee, 1950) was α-estradiol benzoate in peanut oil. The routine procedure was to inject estrogen (85 μg.) subcutaneously for two successive days and to treat with the drugs on the third day. Histologically confirmed rupture or luteinization of follicles and maturation of ova were considered definite evidence of the release of luteinizing hormone.

RESULTS

Atropine-blockade of the lethal actions of acetylcholine-eserine and of epinephrine. The results are summarized in table 1. The readily observed physiological symptoms following the administration of atropine sulfate (30 mg./kg.) include mydriasis and spontaneous fasciculations in otherwise hypotonic muscles. This dosage is above the MLD—it has been lethal to 5 out of 95 rabbits injected during

the past two years. The acetylcholine-eserine solution (450–225 μg./kg.) induced myosis, salivation, spasmodic muscular contractions and death. It is not known that the dosage employed is twice the LD_{100}, but in preliminary experiments death was induced in a few cases with considerably less than half of this dosage (cf. also Markee, Sawyer, and Hollinshead, 1948). In the present experiments 7 animals receiving the 450–225 μg./kg. dose died within a few minutes. However when this dosage of acetylcholine-eserine was immediately preceded by atropine, 14 out of 15 animals not only survived but they failed to show any cholinergic symptoms whatever. The fifteenth

TABLE 1. THE PROTECTION EXERTED BY ATROPINE AGAINST TWICE-LETHAL DOSES OF ACETYLCHOLINE-ESERINE AND EPINEPHRINE

Number of animals	Treatment (intravenously within a minute)	Results		
		Died	Survived	Percentage survived
95*	Atropine (30 mg./kg.)	5	90	95
7	ACh (450 μg./kg.) + Eserine (225 μg./kg.)	7	0	0
15	Atropine, ACh. + Eserine	1	14	93
10†	Epinephrine (1 mg./kg.)	10	0	0
56	Atropine, Epinephrine	27	29	52

* These include animals from Sawyer, Markee and Townsend (1949).
† From Sawyer, Markee and Everett (1950).

rabbit also failed to exhibit cholinergic symptoms, and it is thought that death was probably caused by atropine in this case. As might have been expected from its cholinergic actions, the induction of myosis and salivation (Sawyer, Markee and Hollinshead, 1947), Dibenamine acted synergistically with acetylcholine-eserine. Three rabbits (not listed in table 1) treated with Dibenamine (30 mg./kg.) and the standard dose of acetylcholine-eserine within one minute died during the following minute—more rapidly than without the Dibenamine.

Responses to 1 mg./kg. epinephrine included mydriasis, hypotonia, pulmonary edema and death within a few minutes; this dosage is about twice the LD_{100} (cf. Sawyer, Markee and Everett, 1950). When epinephrine was immediately preceded by atropine, considerable protection was exerted by the latter agent: 52% of 56 rabbits survived the twice-lethal dose of epinephrine.

Such factors as body weight, external temperature, and time between atropine and epinephrine injections were found to affect the degree of protection exerted by the atropine. Under moderate temperature conditions 15/19 rabbits weighing 3 kg. or more died following atropine-epinephrine injection, whereas only 7/29 animals weighing under 3 kg. expired. However, during a period of hot weather in which the day-time temperature of the rabbit room was well over 90° F, 5/8 rabbits in the lighter weight range were killed by the standard atropine-epinephrine dosage.

Atropine-protection against epinephrine is dissipated within 24 hours: five light-weight rabbits under moderate temperature conditions died in response to epinephrine injected 24 hours after the "protective" dose of atropine. On the other hand, epinephrine itself, antagonized by atropine within the preceding minute, seems to exert a protective action against further epinephrine which may last for days. Six rabbits were treated with atropine-epinephrine within a minute and 4 of them were injected with twice-lethal doses of epinephrine at 24 hours, one at 48 hours, and one at 72 hours—all 6 of them survived. A seventh animal which received the second epinephrine injection

TABLE 2. STIMULATION OF LH-RELEASE BY EPINEPHRINE IN THE ESTROGEN-TREATED ATROPINIZED RABBIT

Number of animals	Treatment	Results		
		Hypophysis activated	Failed to activate	Percentage activated
8*	Estrogen	0	8	0
5	Estrogen, Atropine	0	5	0
10	Estrogen, Atropine, ACh-Eserine	1	9	10
7	Estrogen, Atropine, Epinephrine	5	2	71
5	Estrogen, Dibenamine, Epinephrine	0	5	0

* From Sawyer and Markee (1950).

six days later, died. One of the 4 rabbits treated with atropine-epinephrine within a minute and epinephrine at 24 hours received daily injections of twice-lethal dosage of epinephrine for the next 9 days. It not only survived this treatment but gained weight, from 1.8 kg. to 1.95 kg. In these latter experiments it would appear that atropine protects against the initial immediate injection of epinephrine, but that subsequent protection is a function of epinephrine itself.

It is clear from the foregoing results that atropine may temporarily block the lethal effects of epinephrine in rabbits. The question immediately arises as to whether atropine also blocks copulation-induced ovulation by virtue of anti-epinephrine properties. If so, the concept of a cholinergic component in the coital-LH-release reflex (Sawyer, Markee and Townsend, 1949) would lose its only support. If not, the atropine-protected rabbit might respond to large intravenous doses of epinephrine by a release of luteinizing hormone. In either event, the experiments to be presented in the following section were indicated.

Epinephrine-induced release of LH in the estrogen-treated, atropinized rabbit. The results are summarized in table 2. It is apparent that neither estrogen alone (85 μg. estradiol benzoate in oil subcutaneously on two successive days) nor estrogen followed by atropine on the third day stimulated the release of LH. Nor did epinephrine, when preceded within the minute by Dibenamine, stimulate the hypoph-

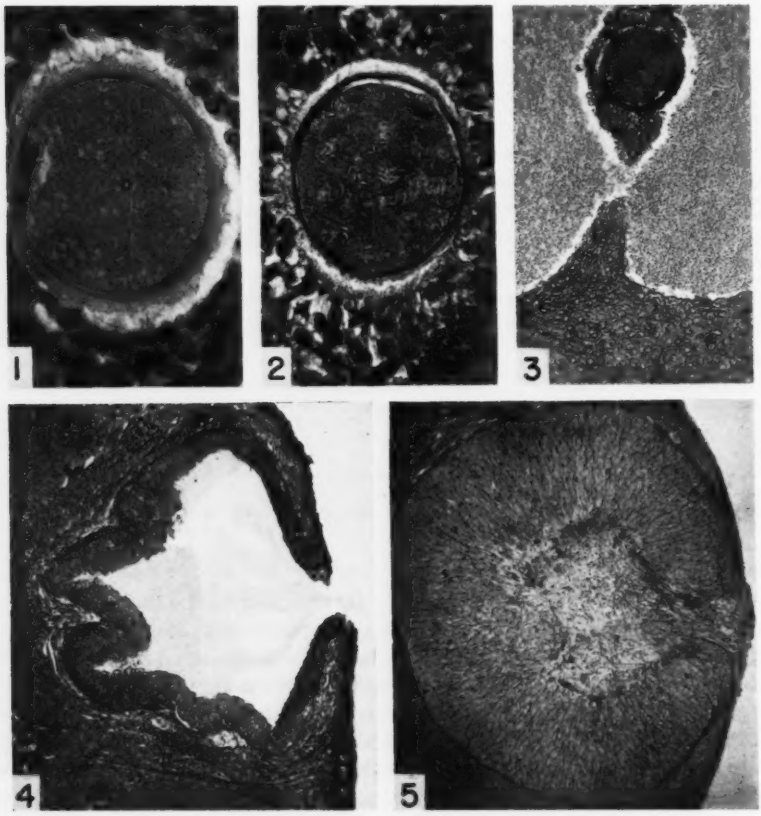

PLATE 1

EXPLANATION OF FIGURES

FIG. 1. Section of an ovum in a hemorrhagic follicle of a rabbit (#592) ovary 48 hours after treatment with atropine-epinephrine. The first polar body is seen in this section and the ovum also contained a second polar spindle. ×300.

FIG. 2. A second polar spindle is visible in this section through an ovum in a hemorrhagic follicle of rabbit #618. An adjacent section contained the first polar body. ×300.

FIG. 3. Luteinization of the granulosa and polar fragmentation of the ovum is apparent in this section from rabbit #622 at 48 hours after treatment. ×150.

FIG. 4. "Ruptured follicle"—a cystic follicle which burst while the ovary was being dissected from rabbit #622. The granulosa shows a somewhat lesser degree of luteinization than fig. 3, from the other ovary of the same animal. ×85.

FIG. 5. Young corpus luteum from rabbit #625 autopsied 72 hours after atropine-epinephrine administration. ×85.

ysis. Acetylcholine-eserine, injected within a minute after atropine, did induce ovulation in one rabbit out of 10. This one rabbit was, however, found to have a pituitary-stimulation mechanism that was highly resistant to atropine: it was also the only rabbit (out of 10) that ovulated in response to mechanical stimulation of the vagina while under the influence of atropine (unpublished). A definite stimulation of LH release was induced by epinephrine in the atropinized rabbit: ovulation or histological evidence of luteinization was observed in 5/7 rabbits.

Two of the 5 positive cases revealed hemorrhagic follicles with ova in various stages of maturation (figs. 1 and 2). Rabbits #592 and #618 had totals of 4 and 3 such follicles, respectively. Maturation phenomena in rabbit ova indicate the release of reduced amounts of ovulating hormone (Pincus and Enzmann, 1935).

The larger follicles of rabbit #622 gave interesting evidence of pituitary stimulation (figs. 3 and 4). One ovary had a large cystic, luteinized follicle which burst while the ovary was being removed (fig. 4). The other ovary had two cystic follicles which were not only well luteinized but their ova revealed "polar fragmentation" (Pincus, 1936). This process, suggesting parthenogenetic development, is characterized by the production of many polar-body-like cells; it rarely occurs *in vivo* in the rabbit (Pincus, 1930).

The other 2 positive cases ovulated, #583 with 7 ruptured follicles and #625 with 5 ruptured and 2 hemorrhagic follicles (fig. 5).

The results summarized in table 2 and illustrated in plate 1 all represented animals that appeared to be in heat at the beginning of the experiment, i.e., at the time of the initial estrogen injection. It was found that the estrogen-atropine-epinephrine treatment would not induce LH-release in rabbits that were initially anestrous (3 cases) pseudopregnant (3 cases) or immature (6 cases). The most desirable conditions of maturity and estrus were found in the weight-group that suffered the most casualties—only 4 out of 19 rabbits weighing in excess of 3 kg. survived the treatment. This consideration kept us from further extension of the estrous-estrogen-atropine-epinephrine series.

DISCUSSION

Little doubt can remain that an adrenergic mediator is employed in the mechanism by which the nervous system controls LH-release from the anterior pituitary. The fundamental evidence for this hypothesis has been summarized earlier in this paper. Not only are the present results consistent with the hypothesis, but in addition, they explain the earlier failure of systemic epinephrine to stimulate LH-release (Markee, Sawyer and Hollinshead, 1948) as due to inadequate dosage.

The present experiments provide strong evidence that atropine

succeeds in blocking the coital stimulus, not by virtue of adrenergic-blocking capacity but by some other mechanism. It is also evident that atropine exerts potent anticholinergic effects within the time limits of its pituitary blocking action. Not only were rabbits protected immediately against the lethal action of high dosages of acetylcholine but, with one exception, the latter injections failed to activate the hypophysis. The exceptional case may be significant; its pituitary-stimulation mechanism appeared to be resistant to atropine, for mechanical stimulation of the vagina induced ovulation in this rabbit following the injection of atropine. Under these unusual conditions, the induction of ovulation in this animal by acetylcholine following atropine strengthens the concept of a cholinergic component in the natural neurogenic stimulus and does not deny that ordinarily atropine blocks the coital stimulus by virtue of its anticholinergic activities.

Evidence has been presented (Sawyer, *et al.*, 1949, 1950) which suggests that atropine-blocked and adrenergic components of the natural stimulus for the release of LH lie in series, with the adrenergic mediator as the final stimulator of hypophyseal cells. The present results are consistent with the order of this sequence. For, if the essential atropine-blocked component were normally to function coincidently with or later than the adrenergic mediator, injected epinephrine would not stimulate successfully in the atropinized animal. Activation by epinephrine in the presence of atropine indicates a lack of dependence of the atropine-blocked activity on the adrenergic mediator. It was impossible to test the converse of this experiment, for acetylcholine was rapidly lethal in the Dibenamine-treated rabbit.

Relatively enormous doses of intravenous epinephrine (1 mg./kg.) appear to be necessary to activate the hypophysis. This fact is in keeping with the suggestion, made elsewhere (Sawyer, Markee, and Everett, 1950), that the natural adrenergic mediator may not be epinephrine itself but some other agent present in commercial epinephrine. The possibility that the natural mediator may be norepinephrine, found by Goldenberg *et al.* (1949) to be present in commercial epinephrine, is being investigated.

The long-enduring protection exerted by injected epinephrine against the lethal effects of further epinephrine is interesting in that most physiological actions of epinephrine are quite transient. The mechanism of the protective action is unknown but one might speculate that, having exerted its adrenergic actions the massive dose of epinephrine secondarily affects the receptive substance as an adrenergic blocking agent. It this connection it may be noted that Eskin (1944) reported that epinephrine blocks the induction of ovulation by estrogen in immature rats, the very mechanism (LH-release) to to which our evidence ascribes a stimulatory action of the drug.

SUMMARY AND CONCLUSIONS

A study employing 91 rabbits reveals that atropine sulfate, in doses adequate to block copulation-induced ovulation, prevents the fatal effects of twice-lethal intravenous dosages of either acetylcholine-eserine (practically complete protection) or epinephrine (survival in about one-half of the cases). The atropine protection against epinephrine poisoning is short-lived, but survivors of atropine-epinephrine administration are protected against the lethal effects of further high doses of epinephrine for days. The prolonged protection is apparently a function of the initial epinephrine itself, acting as an adrenergic blocking agent. These data raise the question as to whether atropine blocks the coital stimulus by virtue of anti-adrenergic properties.

Intravenous epinephrine (1 mg./kg.) stimulated the release of luteinizing hormone in 5 out of 7 mature, estrous, estrogen-treated rabbits protected from death by atropine. The evidence is consistent with the adrenergic nature of the final stimulus in pituitary activation and is strong indication that atropine blocks the copulation stimulus by some means other than its capacity to counteract adrenergic effects.

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INTERFERENCE WITH WOUND HEALING BY THE LOCAL ACTION OF ADRENOCORTICAL STEROIDS[1]

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IN OUR previous studies of the effects of adrenocortical secretions on the anatomy of the body, important changes were observed in fibroelastic connective tissue. Daily treatment of rats with 1 to 3 mg. of adrenocorticotropin for 21 days increased the compactness of the connective tissue fibers of the dermis (Baker, Ingle, Li and Evans, 1948). Subsequently, it was found that direct application of hog adrenal extract to the skin for 108 days caused a marked thinning of the dermis which was limited to the area of treatment (Castor and Baker, unpublished). The matrix of the dermis became more homogeneous, the collagenous fibers appearing to be fused together. Elastic fibers were spared. The fibroblasts and other cellular constituents were reduced in number.

These observations combined with the demonstration that adrenocortical steroids inhibit growth of hair and epidermis by local action (Baker and Whitaker, 1948) induced us to investigate the direct effects of these hormones on the healing of wounds. More recently Ragan, *et al.* (1949) reported that administration of large doses of cortisone to rabbits retards the rate of repair of wounds and formation of granulation tissue. Likewise, treatment of human patients with adrenocorticotropin interferes with the formation of granulation tissue in open wounds (Ragin, Grokoest and Boots, 1949). Overdosage with cortisone or ACTH causes a negative nitrogen balance. Therefore, the studies of Ragan and his colleagues leave unanswered one important question: Does this retardation in growth of tissue result from a generalized disturbance of protein metabolism as indicated by the excessive excretion of nitrogen, or may adrenocortical hormones inhibit healing of wounds by direct action on the peripheral tissues? If a general non-availability of nitrogen for protein synthesis is necessary, and if other organs are involved, the possibility is increased that this effect and other physiological and histological modifications elicited by treatment with high doses of adrenal steroids are non-specific. Therefore, it is most important that an answer to this question be found. Our report will demonstrate that

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retardation in the formation of granulation tissue may be induced by the local action of adrenal steroids, thus eliminating the possibility that the effect must be preceded by a general disturbance of protein metabolism.

MATERIALS AND METHODS

Four experiments were carried out on adult rats of the Long-Evans strain which were fed ad libitum. All animals were kept in individual cages. The skin covering the dorsal half of the neck was chosen for study. In experiment 1, the neck caudal to the right ear of 12 rats was treated percutaneously with daily doses of 0.1 cc. of natural 11-dehydro-17-hydroxycorticosterone (cortisone) in 25% alcohol (1 mg. of hormone per cc.). Treatment was continued for an average of 24 days. Then, a piece of skin was removed with a No. 6 Keyes' skin punch in symmetrical locations on each side of the neck, the right wound being located in the area of hormone application. The pieces of skin excised measured 6 mm. in diameter. Treatment was continued as before with the wounds being measured on alternate days of the experiment. Five control rats were treated similarly except that 25% alcohol was substituted for the solution of hormone.

In experiment 2, a similar procedure was followed except that hog adrenal extract[2] was used and larger pieces of skin measuring 14 mm. in diameter were excised by means of scissors after a circle had been marked on the skin with India ink. The pre-treatment period lasted for an average of 67 days. The wounds were measured frequently during the experiment and their area calculated by the formula $\pi ab/4$ where a and b represent the longest and shortest axes. Fourteen rats were treated with the extract and 9 with alcohol.

In experiment 3, the procedure was similar to that of experiment 2 except that the right wounds were treated twice daily with the extract, thus doubling the total daily dose. On the seventh day, each wound in addition to some adjacent skin was removed, divided into halves, flattened on pieces of cardboard and fixed for histological study in chilled 80% alcohol, 4% lead acetate, Bouin's and Carnoy's fluids. In this group, 7 male rats were treated with the extract and 6 with 25% alcohol. Sections of these specimens were stained with hematoxylin or methylene blue and eosin, iron hematoxylin and the Masson procedure, Hotchkiss' (1948) periodic acid-leucofuchsin, Gomori's procedures for reticular fibers (1937) and alkaline phosphatase (1939) and with 1% aqueous toluidine blue.

The procedure of experiment 4 was similar to that used in experiment 3 except that the animals were not pre-treated with the extract before the wounds were made. Extract was administered to 3 rats and 25% alcohol to 3 controls.

RESULTS

Experiment 1. The time of closure of the right wound of some of the rats treated with cortisone was retarded slightly as compared with that of the left side. However, in a number of the cortisone-

[2] We wish to thank Drs. M. H. Kuizenga and W. J. Haines of The Upjohn Company for this extract. It was prepared in 25% alcohol with activity by the liver glycogen test equivalent to 1.0 mg. of cortisone per cc.

treated rats there were no differences between the two sides so that these results were not considered significant.

Experiment 2. Although the pieces of skin removed from each side of the neck were of equal size, immediately after the operation the right wound opened up wider than that on the left side. This is shown by the greater average area of the right wounds 2 days after the operation (fig. 2). This difference is due to the changes induced in the dermis by the extract during the period of pre-treatment. Prolonged

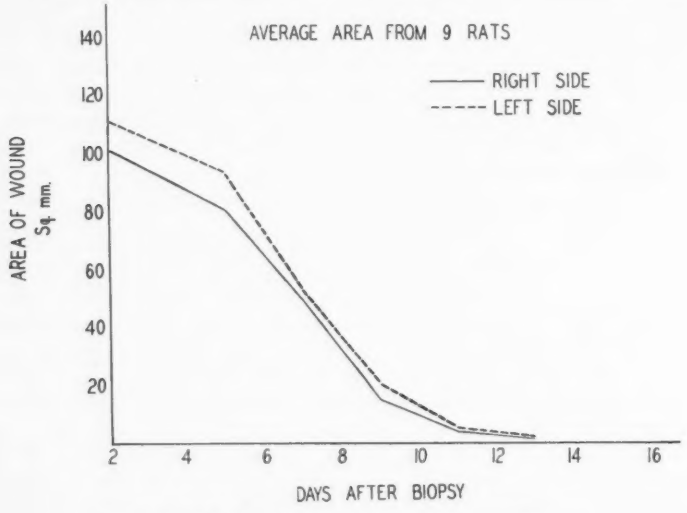


FIG. 1. Effect of Alcohol on Rate of Wound Closure.

local treatment with this extract causes thinning of the dermis accompanied by atrophy of collagenous fibers. The elastic fibers are spared and even become more concentrated (Castor and Baker, unpublished). These changes in the microscopic structure of the surrounding skin probably account for the greater area of the wounds made in the skin to which extract had been applied. It may be seen from figure 2 that healing of the right wound proceeded in spite of continued treatment, although the right one lagged behind that of the left side. In some cases, there was a striking difference in the time of closure (fig. 4), but when the average area of the wounds of the extract-treated animals on successive days of the experiment were plotted against time (fig. 2), it was seen that the time of complete closure was not greatly different on the two sides. It must be pointed out that it was difficult to determine by gross observation exactly when healing was completed, especially if the wound was covered with a crust. Therefore, the magnitude of the retardation may have been greater than was realized. We concluded that this extract, when

so applied, could not counteract completely the stimulation to growth around the treated lesion caused by cutting the skin. This stimulation was manifested also by the re-initiation in growth of hair around the wound, even though treatment with the extract was continued. In the neighborhood of the right wound shown in figure 4 is seen the melanotic pigmentation of the skin which accompanies reactivation of hair follicles.

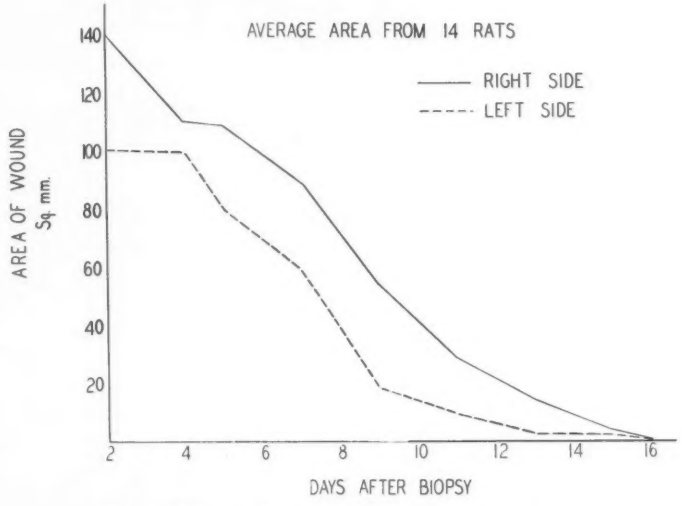
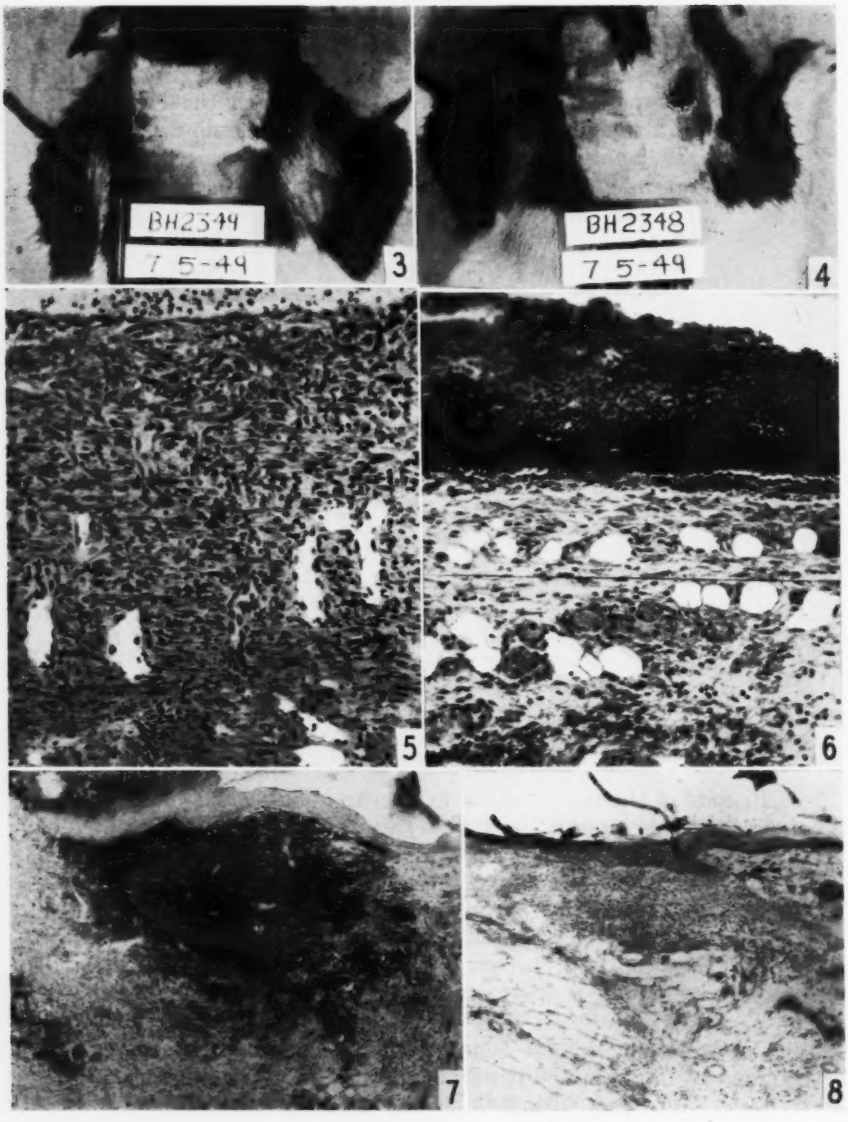


FIG. 2. Effect of Extract on Rate of Wound Closure.

In spite of the failure of the extract to hold healing completely in abeyance, some interference with repair was indicated by the different appearance of the wound treated with extract as contrasted to the left one on the same rat or to those of control animals treated with alcohol. The crust which formed over the extract-treated wound was thin, light tan in color and slightly concave. This contrasted to the thicker crust of dark brown color which characterized the non-treated and alcohol-treated wounds. It was inferred that the adrenal hormones had interfered with the formation of exudate and granulation tissue. Also, the cut edges of the skin around the wounds receiving extract remained flat and thin, failing to show the usual contracture. Treatment with 25% alcohol did not retard the rate of healing (figs. 1 and 3).

Experiment 3. This experiment was designed to demonstrate the action of adrenal steroids on the histology of wound repair before stimulation to growth caused by cutting the skin had taken full effect.

For six days after the operation, formation of granulation tissue



EXPLANATION OF FIGURES

FIG. 3. Control rat for BH 2348 of figure 4. The right wound had been treated daily with 0.1 cc. of 25% alcohol for 12 days. There is no difference in the amount of healing which has occurred on the two sides. The pattern of the hair which grew during the preceding week is symmetrical.

FIG. 4. This rat was treated daily behind the right ear with 0.1 cc. of hog adrenal extract (equivalent to 100 μg. of cortisone) for 50 days during which time growth of

was impaired greatly by the extract. Figure 5 shows the extensive proliferation of fibroblasts and formation of new blood vessels in the center of the left wound of a rat whose right wound was treated with extract. Fibroblasts in this and other areas were large and stellate with considerable cytoplasmic basophilia. Mitoses were frequent. Reticular fibers formed a loose network throughout the granulation tissue. After treatment with the extract, growth of granulation tissue in the center of the wound was stopped completely (fig. 6). Only a thin layer of dense connective tissue with small flattened, non-basophilic fibroblasts over-laid the scattered subcutaneous adipose cells. Most of the fibers were collagenous in type, only a few reticular fibers being present. Fibroblasts were not proliferating. There were few new outgrowths of blood vessels. A few of the vessels were surrounded by accumulations of small fibroblasts, between which were newly-formed reticular fibers. These cells and fibers were much more compactly arranged than was true of the granulation tissue of the left side.

The cut edge of the skin in the extract-treated wounds presented a more variable histology. In some locations, no proliferation of connective tissue had occurred, whereas, elsewhere it had begun but was not as prolific or as well vascularized as around the left wound. This limited growth usually started beneath the sheet of thickened stratified squamous epithelium which was spreading out over the wound. Densely arranged fibroblasts and intricate networks of delicate reticular fibers were present in this area. The extract also caused a marked reduction in alkaline phosphatase activity at the periphery of the lesion (figs. 7 and 8). In the wounds which were treated with alcohol or nothing, the enzyme was present in fibroblasts, capillary walls and the interstitial matrix. Although normal dermis shows little phosphatase activity, Fell and Danielli (1943) demonstrated that in the healing of wounds in rat skin, this enzyme reaches a high peak of activity at the time when formation of collagen begins.

Experiment 4. Without pre-treatment of the skin with extract, growth of granulation tissue was impaired but to a somewhat lesser degree than with pre-treatment.

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hair had been inhibited in the area of treatment for 36 days. Then, wounds of equal size were made on each side. Treatment of the right wound was continued for 12 days. Closure is delayed. Re-initiation of hair growth is indicated around the right wound by the presence of cutaneous pigmentation.

FIG. 5. Much granulation tissue has formed in the center of the left wound 7 days after the wound was made. Iron hematoxylin and Masson. ×140.

FIG. 6. Granulation tissue has failed to form in the center of the right wound of the same rat as that illustrated in figure 5 as a result of treatment twice daily for 7 days with adrenal extract (equivalent to 200 μg. of cortisone a day). Technique as in figure 5.

FIG. 7. Alkaline phosphatase is quite active at the periphery of the left wound of a rat treated in the same manner as that shown in figure 5. Gomori procedure, sodium glycerophosphate substrate, incubation for 24 hours at 37°C. ×60.

FIG. 8. Alkaline phosphatase activity is reduced at the periphery of the right wound of the same rat as that shown in figure 7. Technique the same as in figure 7.

DISCUSSION

Abundant evidence supports the concept that increasing the concentration of circulating adrenal steroids above the normal level will cause retardation in growth of the body, this effect being reflected in many different tissues (Baker, 1950). These changes are induced most easily in the more rapidly proliferating tissues but ultimately affect the connective tissues also. The demonstration that adrenal steroids retard growth of hair and epidermis (Baker and Whitmaker, 1948), cause atrophy of dermal connective tissue (Castor and Baker, unpublished) and impair growth of granulation tissue by local action, shows clearly that these effects are not predicated upon a loss of nitrogen from the body. It is more probable that the action is directly on the peripheral cells and even on the interstitial matrix of the connective tissues.

The successful retardation in growth of granulation tissue in the rat by the local action of adrenocortical steroids is particularly interesting in the light of the failure of Ragan, *et al.* (1949) to secure this effect by the daily injection into rats of 1 mg. of cortisone per 100 gm. of body weight. Possibly, by direct application one can build up a high concentration of the hormone in a limited area more easily than by systemic injection.

In view of the dramatic relief which treatment with ACTH or C-11 oxygenated adrenocortical steroids affords patients suffering from certain diseases involving connective tissue, it is important to discover in our studies which element of connective tissue is affected primarily. There are at least three possible foci of action, namely, fibroblasts, collagenous fibers and ground substance. Our observations show clearly that adrenal extract prevented the proliferation of fibroblasts and of the endothelium of capillaries for a limited period of time. Fibroblasts which were already present in the connective tissue failed to reach the size of those found in the growing granulation tissue of the non-treated wounds. Hence, under the conditions of this experiment, the primary site of action of these compounds was on the fibroblasts. This conclusion does not preclude the possibility that the adrenal steroids might have modified collagenous fibers or ground substance if they had been formed.

SUMMARY

Direct application of hog adrenal extract dissolved in 25% alcohol to cutaneous wounds in the rat caused some delay in closure of the wounds, but the treatment did not counteract completely the stimulation to growth caused by cutting the skin. As determined by the liver glycogen test, the daily doses were equivalent to 100 or 200 μg. of cortisone. Nevertheless, a striking interference in formation of

granulation tissue resulted from this treatment, the effect being limited to the area of treatment.

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THE *IN VITRO* REACTION OF CELLS TO ADRENAL CORTICAL STEROIDS WITH SPECIAL REFERENCE TO LYMPHOCYTES[1]

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THE REACTION of lymphocytes to adrenal cortical steroids in vitro has been studied recently with contradictory results. Robertson (1948) and Delaunay, Delaunay and Lebrun (1949) reported no effects of adrenal cortical compounds on lymphocytes, while Schrek (1949) and Hechter and Johnson (1949) described a slight but definite toxic action.

The work to be reported is a study of the action *in vitro* of adrenal cortical extracts, compound A, compound E and desoxycorticosterone upon lymphocytes, marrow and testicular cells, white cells of the peripheral blood and peritoneal washings of rabbits which consist mostly of polymorphonuclear neutrophiles (pseudoeosinophiles).

METHOD

Schrek's (1947) method of unstained cell counts, with some modifications, was employed. In brief, the technique consisted of making a suspension of cells in homologous serum or plasma, or in physiological salt media. A single animal provided both the serum or plasma and the cells, except in the experiments on human material in which the cells were derived from lymph nodes from fresh autopsies and the plasma was taken from the same Type O donor. Tissues of rats, rabbits, guinea pigs and human beings were used.

Fragments of thymus, lymph nodes, spleen, bone marrow and testis were teased in a small amount of serum or plasma, or salt solution. This preparation was then passed through a disc of 80 gauge monel wire gauze to eliminate stroma and large tissue particles. Peripheral white blood cells were collected by centrifugation in a specially constructed tube which permitted the formation of a long narrow column of buffy coat. The buffy coat was removed and resuspended in serum or plasma. Neutrophiles were harvested by injecting rabbits with approximately 300 cc. of saline intraperitoneally. Three hours later about 50–60 cc. of the fluid was removed and centrifuged. The supernatant was poured off and the sedimented cells resuspended in homol-

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ogous serum or plasma. The number of cells per cmm. was adjusted to the desired level by adding more serum, plasma or salt solution.

Two control mixtures were studied simultaneously in all experiments in which oily products were tested. One control mixture consisted of cell suspension plus the vehicle in which the hormone was suspended, i.e. cotton seed oil containing 1% chlorbutanol, or sesame oil. The second control consisted of cell suspension in a physiological salt solution such as Gey's medium, Tyrode's or simply physiological saline. In experiments in which the crystalline product was added directly to the suspension, the control was the suspension itself without addition of any material.

To a measured amount of suspension, usually 0.4 ml., various reagents were added. The reagents used were: Lipo-Adrenal Cortex, in dosages of 0.1 ml. to 0.2 ml., except in tests to determine the highest effective dilution[2]; aqueous adrenal cortical extracts in dosages from 0.5 ml. to 5.0 ml.[3]; crystalline compound A (dehydrocorticosterone acetate) in dosages from 2 mg. to 4 mg., except in experiments to determine the minimal effective dosage[4]; crystalline compound E (11-oxy-17-hydroxycorticosterone acetate) in dosages from 2 mg. to 4 mg.[5]; desoxycorticosterone in dosages from 2 to 5 mg.[6]; testosterone propionate in dosages from 2 to 5 mg.[7] Other non-steroid compounds were also employed: insulin,[8] crystalline ACTH[9] and purified growth hormone.[10]

Before each count the cell-hormone, cell-oil and cell-salt solution mixtures were shaken for at least 5 minutes on a shaker rotating through a 90 degree arc at 120–150 times each minute. For counting, the mixture was taken up to the 05 mark of a white cell pipette and diluted to the 11 mark with a 1:2,000 solution of eosin in physiological salt solution. The salt solutions employed were 0.85% saline, Tyrode's and Gey's. White cell pipettes containing cells and eosin diluting agent were shaken for 5 minutes on the shaker before a drop was placed in the counting chamber.

For purposes of calculation the first count made on each mixture was considered the baseline, or 100%, of the total number of cells per cmm. All counts thereafter were expressed as a percentage of this first determination. The number of non-viable cells as detected by staining with eosin was expressed as a percentage of the total of each individual count. All counts were done by a single observer and on the same counting chamber. Each count was an average of the number of cells on both sides of the chamber, the two halves agreeing within 15%. In addition to the total number of cells the number stained with eosin was also ascertained.

According to Schrek (1947) and Pappenheimer (1917), a viable cell is not permeable to stain and appears in the counting chamber as a colorless,

[2] Upjohn's.

[3] Wilson's and Upjohn's.

[4] Merck. This material was kindly supplied by Dr. C. N. H. Long, Yale University, School of Medicine.

[5] Merck. This material was kindly supplied by Dr. C. N. H. Long, Yale University, School of Medicine.

[6] Roche-Organon, Doca: Ciba, Percorten.

[7] Rare Chemicals Inc.: Ciba, Perandren.

[8] Burroughs-Wellcome.

[9] Armour & Co.

[10] The growth hormone was kindly supplied by Dr. Alfred Wilhelmi, Department of Physiological Chemistry, Yale University School of Medicine.

slightly refractile sphere. A non-viable cell takes up stain and appears pink or red with eosin dye.

A sterile technique was used throughout, although this apparently was not necessary since bacterial contamination did not influence the outcome over the period of observation.

RESULTS

1. Reaction of Cells to Lipo-Adrenal Cortex

Lipo-Adrenal Cortex had a dramatically rapid toxic action upon lymphocytes derived from thymus, lymph nodes or spleen. Numerous

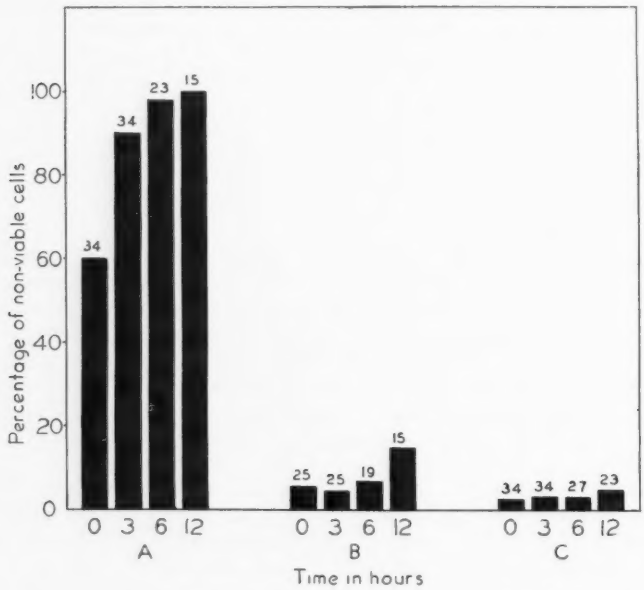

FIG. 1. Effect of Lipo-Adrenal Cortex on Cell Suspensions. A. Mixture of extract and cell suspension. B. Mixture of cotton seed oil and cell suspension. C. Plasma suspension alone. All mixtures incubated at room temperature. Figures above columns show number of observations.

examinations of cell suspensions mixed with this extract revealed that by the time the baseline count was made, (at 0 hours), an average of 60% of the total number of cells was already stained. Baseline counts determined within 5–10 minutes after the Lipo-Adrenal Cortex was added to the suspension generally showed a much lower percentage of non-viable cells, between 2–20%, while mixtures which were examined 30 minutes after the preparation was made presented between 80–100% stained cells. Within three hours, on the average, 90% of the total cell count was eosin-stained and at 6 hours almost 100% of the cells were non-viable (v. fig. 1 A).

Control mixtures derived from thymus, in contrast, maintained a low percentage of non-viable cells over a period of 12 hours. From the baseline count up to 6 hours the percentage of eosin-stained cells ranged from 2–6% and reached only 15% at 12 hours of incubation (v. fig. 1 B & C). Control suspensions derived from lymph nodes or spleen fragments showed at the initial examination between 20–30% eosin-stained cells. This percentage was constant up to 6 hours and reached 45% at 12 hours.

Lipo-Adrenal Cortex injured other cell types just as rapidly as lymphocytes. Marrow elements (rat and rabbit), testicular cells (rat), peripheral white blood cells (rat and human), and pseudoeosinophiles harvested from peritoneal washings (rabbit) became stained as quickly and to the same degree as lymphocytes. Control suspensions of these cell types showed less than 20% nonviable cells over the period of observation, except testicular suspensions, in which the baseline and subsequent counts revealed between 45–55% stained elements.

In order to test whether serum or plasma was required to achieve this effect of Lipo-Adrenal Cortex, cell suspensions were made in plasma from adrenalectomized rats five to ten days after operation. The result was no different from the above. In addition, cells were freed of plasma by three to four washings in Tyrode's or Gey's media with alternate centrifugation, and resuspended in the salt solution to which extract had been added. The hormone produced the same dramatically rapid effects as observed above.

It should be mentioned, however, that cells freed of plasma and suspended in various physiological media are apparently somewhat injured, for they show a very faint yellow-orange hue when diluted with the eosin-containing fluid. Further, these cells were lysed by mechanical shaking more readily than those prepared in serum or plasma. It was found that at least 2 parts of serum or plasma must be added to 5 parts of the salt solution in order to prevent the faint coloration of cells.

2. Reaction of Cells to Mechanical Shaking and Temperatures of Incubation

Shaking a mixture of cell suspension and Lipo-Adrenal Cortex 120–150 times per minute resulted in disintegration and disappearance of cells. Within three hours the total cell count dropped from 100% to approximately 40%, and at 12 hours, on the average, only about 10% of the cells were found (v. fig. 2 A). The original number of cells per cmm. was not material, for the same result was obtained through a range of from 350,000 to 2,000 cells per cmm. On the other hand, control mixtures, consisting of cell suspension plus oil or salt solution, showed no diminution in total cell count, and in fact very often increased in numbers, apparently from proliferation (v. fig. 2 B).

Thymus cells in plasma or serum, without addition of cortical steroids and kept at room temperature, fluctuate in numbers despite extremely careful and repeated chamber counts on the same suspension. In slightly better than half of all the control mixtures, the counts, within the first 12 hours and subsequent to the first, showed an increase. The rise in some instances was as high as 30% beyond the original determination. After 12 hours there was a gradual and slight diminution of cells so that at 24 hours the total number returned to

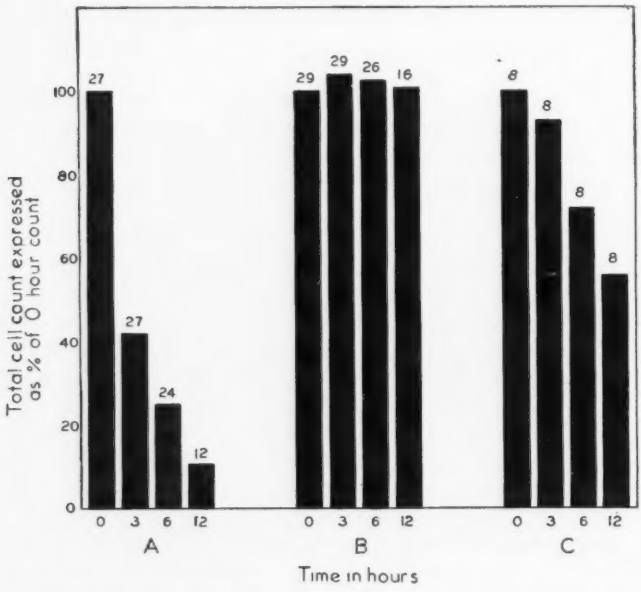

FIG. 2. Effect of Mechanical Shaking on Cell Lysis. A. Mixture of Lipo-Adrenal Cortex & cell suspension, shaken 150 times per minute. B. Cell suspension with and without cotton seed oil, shaken 150 times per minute. C. Mixture of Lipo-Adrenal Cortex and cell suspension. No shaking. Figures above columns show number of observations.

the baseline level or slightly below. In control mixtures which showed no increase in cell numbers the count remained stable for 12 hours and then gradually declined to approximately 80% of the baseline count at 24 hours. Control mixtures incubated at 37.5 degrees C did not increase over the initial count beyond a 10% technical error.

The rise in cell counts seemed most likely to result from cell proliferation rather than from faulty technique. Mitoses were seen on occasion in the counting chamber and more often in fixed stained smears of incubated mixtures.

In mixtures of cell suspension and Lipo-Adrenal Cortex which were allowed to stand, without shaking, the rate of disappearance of

cells (i.e., the diminution of the count below the baseline determination) proceeded much more slowly than in those mixtures which were agitated. At 3 hours the total number of cells decreased only to 93% of the first count; at 6 hours to about 72%; and at 12 hours to about 56% (v. fig. 2 C).

Temperature had a marked effect on cell suspensions. At room temperature (25–26 degrees C) without the presence of Lipo-Adrenal Cortex, lymphocytes and other cells were quite stable with respect to eosin staining up to 24 hours. In this period of time the percentage of stained cells rose from 3% to 8% (v. fig. 3 B). At 37.5 degrees C the percentage of eosin-stained cells began to increase after 3 hours

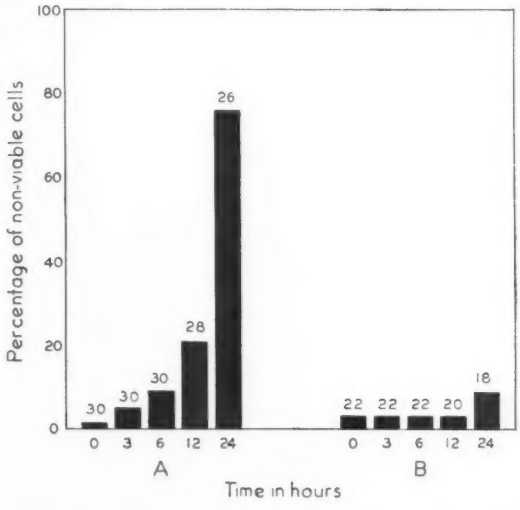

FIG. 3. Effect of Temperature on Cell Lysis. A. Lymphocyte suspension incubated at 37.5 degrees C. B. Lymphocyte suspension incubated at room temperature (25–26 degrees C). Figures above columns show number of observations.

of incubation and rose slowly over 24 hours from 1–2% at 0 hours to 75% at 24 hours (v. fig. 3 A). At the same time the total cell count diminished more rapidly than in mixtures kept at room temperature, from 100% to about 30-40% of the baseline count at 24 hours.

In brief, mechanical shaking lysed non-viable cells but did not have any apparent effect on viable unstained cells. Increased temperature accelerated the death and disintegration of cell elements.

3. Reaction of Cells to Aqueous Cortical Extracts

Wilson's and Upjohn's aqueous adrenal cortical extracts were used. The former is prepared in physiological saline and the latter in physiological saline containing 10% alcohol. For controls, cell suspen-

sions were mixed with physiological saline and saline with 10% alcohol.

Physiological saline, as well as other salt media, caused cells to show faint yellow-orange coloration when diluted with eosin and to disintegrate more readily with agitation. Serum or plasma apparently protected cells from this; at least 2 parts of serum or plasma to 5 parts of salt medium were needed to prevent this alteration. It is of prime importance to examine control mixtures simultaneously with

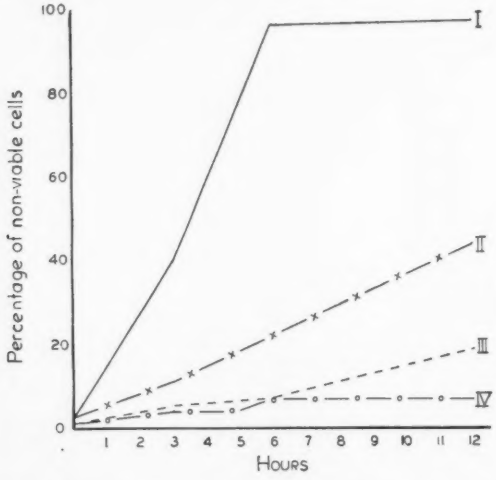

FIG. 4. Effect of Aqueous Adrenal Cortical Extract on Cell Suspension. I. Extract and suspension incubated at 37.5 degrees C. II. Physiological saline and suspension incubated at 37.5 C, control for I. III. Extract and suspension incubated at room temperature. IV. Physiological saline and suspension incubated at room temperature, control for III.

experimental mixtures to observe the specific action of the extract and to separate this action from that of the salt media.

Under these circumstances, 5 ml. of Wilson's aqueous cortical extract mixed with 2 ml. of cell suspension in serum or plasma showed a significant effect (v. fig. 4 III). The percentage of eosin-stained cells rose slowly from 2.5% to about 20% after 10 hours of incubation at room temperature. The control mixtures showed a rise of non-viable cells from 1–2% to only 10% (v. fig. 4 IV). At 37.5 degrees C the effect was more pronounced. The percentage of stained cells increased from 1–2% to 95% in 10 hours of incubation with the extract, while the control showed a rise from 1–2% to 44% during this same period (v. fig. 4 I & II). As the number of non-viable cells increased the total cell count decreased, the fall being greater in the vials with hormone than in the controls. At no time, however, did the stainability or disappearance of cells ever approach the effect of the Lipo-Adrenal

Cortex in rapidity of action. Less than 5 ml. of Wilson's extract added to lymphocyte suspensions was without significant effect.

No satisfactory results were obtained with Upjohn's aqueous extract because the hormone is prepared in 10% alcohol which is noxious to lymphocytes.

4. Reaction of Cells to Other Steroids and Compounds

A variety of other steroids was incubated with lymphocyte suspensions and of these only compound A had any effect. Two mg. of

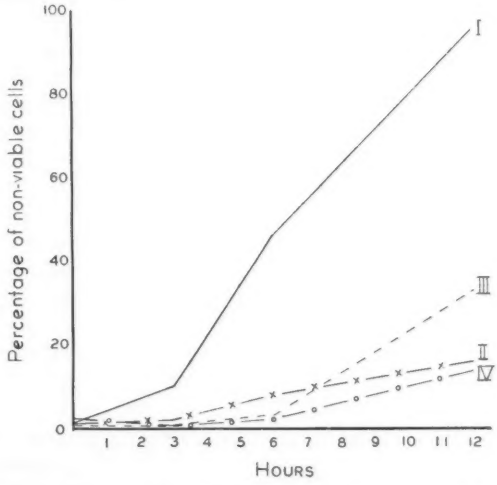

FIG. 5. Effect of Compound A on Lymphocytes. I. Compound A and lymphocyte suspension incubated at 37.5 degrees C. II. Lymphocyte suspension alone incubated at 37.5 degrees C., control for I. III. Compound A and lymphocyte suspension incubated at room temperature. IV. Lymphocyte suspension alone incubated at room temperature, control for III.

compound A (dehydro-corticosterone acetate) added to a plasma suspension of lymphocytes caused the percentage of non-viable cells to rise from 1–2% to 95% within 6–12 hours of incubation at 37.5 degrees C (v. fig. 5 I). Control mixtures, consisting of a plasma suspension alone, showed an increase of stained cell from 1–2 % to 15%, during the same period (v. fig. 5 II). At room temperature the effect of compound A was much less rapid, rising from 1–2% at 0 hours to 25–30% at 12 hours, and almost to 100% at 20 hours, while the controls maintained a level of non-viable cells at about 2–4% for 20 hours (v. fig. 5 III & IV).

5. Minimal Effective Dosage of Lipo-Adrenal Cortex and Compound A

The minimal effective dosage was arbitrarily defined as the highest dilution of Lipo-Adrenal Cortex or the least amount of compound A

which produced 90% or more stained cells in 12 hours or less of incubation.

Each ml. of Lipo-Adrenal Cortex contains 40 rat units according to the manufacturer. Dilution of this extract to 0.8 rat units consistently caused staining of 90% or more of the cells in 0.4 ml. of suspension within 12 hours of incubation at 37.5 degrees C. Dilution to 0.65 rat units yielded inconsistent results, at times satisfying the criteria of a minimal effective dose and at times not.

Significant effects were obtained with as little as 0.05 of a rat unit but the percentage of non-viable cells at 12 hours ranged from 17–23% in contrast to 5–8% for control mixtures.

Compound A was dissolved in plasma by shaking and heating to 37.5 degrees C and appropriately diluted with further addition of plasma. The minimal effective dosage of this material was found to be 80 μg. Significant effects were noted with as little as 40 μg. but at this level only 42% of the cells in suspension were non-viable at 12 hours of incubation at 37.5 degrees C in contrast to 20% stained cells in the control vial.

Compound E (11-oxy-17-hydroxy-corticosterone acetate), desoxycorticosterone, testosterone propionate, insulin, ACTH and growth hormone were tested and found to have no action on lymphocytes.

6. Characteristics of the Cells Incubated with Lipo-Adrenal Cortex or with Compound A

A description of the cytological features of lymphocytes suspended in plasma or serum over a period of time has been adequately provided by Schrek (1947).

Lymphocytes, pseudoeosinophiles, marrow elements or spermatogonia mixed with Lipo-Adrenal Cortex or compound A began to swell very early, sometimes to a diameter ten times their original size. The nucleus was no longer visualized and it blended with the cytoplasm, the entire cell being a pink or red sphere. The cytoplasmic edge became blurred and indistinct. Some of the cells became ghost forms, displaying a refractile blurred edge and a pale smooth interior. Others showed a densely stained dot within the swollen cell from which radiated fine refractile lines. In the counting chamber many cells were seen to burst, disintegrate and fade away while others fragmented before they enlarged very much. Cells incubated with the extract and absorbed in a gelfoam sponge, fixed, embedded in paraffin, sectioned and stained, under the microscope were pycnotic, not swollen or enlarged. A good deal of nuclear debris was also present.

DISCUSSION

The above observations demonstrate that Lipo-Adrenal Cortex is a general cytoxic agent whose action is almost immediate and ex-

plosive. Compound A has a similar action on lymphocytes though its effects are not so rapid. The effect is first noted as a diffuse eosin-staining of the cell; this is followed by disintegration of the cell membrance and lysis, a process accelerated by mechanical shaking.

These *in vitro* results correspond closely in time sequence with the lymphoid dissolution and peripheral lymphopenia observed *in vivo* (Dougherty and White, 1944, 1945, 1947). The lethal effects of adrenal extracts on other cell types, except eosinophiles (Speirs & Meyer 1949), have not been described, although this action is as rapid and dramatic as that on lymphocytes. Also in line with *in vivo* studies on lymphocytes is the fact that desoxycorticosterone and testosterone propionate have no apparent influence on this or other cell types *in vitro* (Forsham *et al.*, 1948; Thorn *et al.*, 1949).

Compound E, reported by several investigators to cause lympholysis *in vivo* (Wells and Kendall, 1940; Forsham *et al.*, 1948; Thorn *et al.*, 1949) and to be much more potent than compound A, showed no lympholytic action by the present methods. It is possible that the 11-oxy-17-hydroxy-corticosterone, which is not readily soluble in plasma and other aqueous media, was unable to act upon lymphocytes because of this property, in contrast to compound A which is more readily soluble.

The failure of Robertson (1948) to notice any effect of eschatin and eucortin, aqueous cortical extracts, on lymphocyte suspensions *in vitro* may be ascribed to several factors. These commercial products are much less potent than is Lipo-Adrenal Cortex. Secondly, he did not examine the cell suspensions for viability nor did he use a shaker, which would have lysed non-viable cells. The total number of cells only was determined, and without agitation there would be a comparatively slight diminution of cells from the original determination.

Delaunay *et al.* (1949) found no effects on lymphocytes incubated with 11-dehydrocorticosterone. They used neutral red as an indicator for viability and their method of preparation of cells was different. These facts may explain the variance of observed results.

Schrek (1949) reported that adrenal steroids had only a mild toxic effect on lymphocytes and none at all on marrow elements. In the present work Lipo-Adrenal Cortex acted almost immediately and explosively, not only on lymphocytes but upon other cell types as well. This discrepancy in observations may be due to the much higher dosages used here as against the microgram amounts employed by Schrek.

The observations of this work do not corroborate the report of Hechter and Johnson (1949) that lymphoid and brain tissue potentiate the action of Lipo-Adrenal Cortex on lymphocytes. Lipo-Adrenal Cortex can produce eosin-staining in lymphocytes and other cells rapidly without the presence of any tissue other than the cells themselves. Several unpublished experiments indicate that brain tissue

actually inhibits the toxic action of the extract on suspended lymphocytes. Furthermore, the extract has a lethal action upon cells suspended in Gey's or Tyrode's medium, without the presence of serum or plasma, and the action is of the same order of rapidity. It is true, as Hechter and Johnson observed, that serum or plasma has a protective effect on lymphocytes, but the protection is not against the action of the Lipo-Adrenal Cortex but against the toxicity of salt solutions as shown by the data presented.

SUMMARY

Lipo-Adrenal Cortex is a general cytotoxic agent which rapidly and completely injures, *in vitro*, lymphocytes, marrow and testicular cells, pseudoeosinophiles and peripheral white blood cells. Compound A has a similar but somewhat less rapid action upon lymphocytes. Aqueous adrenal cortical extract is much less potent in damaging cells, requiring larger doses and longer intervals of time for its effect. Non-viable cells are readily lysed by agitation which has no apparent effect on viable cells. Increase of the temperature of incubation increases the rate of non-viability and disintegration of cells in the absence of agitation. Compound E, desoxycorticosterone, testosterone propionate, growth hormone, ACTH, and insulin are without effect on cells as evidenced by staining with eosin.

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FOLLICLE-STIMULATING HORMONES OF THE ANTERIOR PITUITARY OF THE SHEEP AND THE HOG

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RECENTLY, Li, Simpson and Evans (1949) described the isolation of follicle-stimulating hormone from sheep pituitary glands. Work on the same problem with hog glands as starting material has been under way in our laboratories for several years without, however achieving complete purification of the hormone. In the case of another anterior pituitary gonadotrophin from the same two species (metakentrin, ICSH, LH) it was clearly shown that although the endocrine effects of the two were identical, they were not the same proteins but had widely different isoelectric points, molecular weights and antigenic properties (Shedlovsky, *et al.*, 1940; Li, *et al.*, 1940 (c), 1942; Chow, *et al.*, 1942; Gurin, 1942). It was therefore of interest to compare hog and sheep follicle-stimulating hormones. The purpose of this communication is to furnish experimental evidence for the conclusion that here again a different protein is obtained from each of the two species although the biological effects of the two hormones are identical and they are much more alike chemically than the interstitial-cell stimulating hormones (metakentrins). Procedures for extracting purified follicle-stimulating hormone from hog pituitary glands are also discussed.

METHODS

All assays were performed with hypophysectomized immature male or female rats. In the assays with female rats the method of Li, Simpson and Evans (1949) was followed: hypophysectomy was performed in females 27 days old; one week later, one-third of the total dose of hormone was administered once daily for 3 days and necropsy was performed 24 hours after the last dose; when a large dose of hormone was administered, injections were made once daily for 4 days with necropsies on the fifth day. The ovaries, uterus and adrenals were sectioned for microscopic examination. Male rats were hypophysectomized at an age of 21 days and injections were begun 24 hours later. One-fourth of the total dose was injected once daily for 4 days and the rats were sacrificed one day later. Provided that a homogeneous

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group of rats was used for various doses of a given preparation of follicle-stimulating hormone of high biological purity, there was a linear relationship between the average weight of the testes and the logarithm of the quantity of hormone administered which ranged from the minimal effective total dose (0.05 mg.) to a dose thirty times as great.

To test the qualitative (biological) purity of hormone, very large doses were employed. In the hypophysectomized female rats, the largest dose was at least 20 times (2.0 mgm.) that which had a detectable effect. Particular attention was paid to the adrenals and uterus in females in order to detect contamination by adrenal corticotrophic hormone (ACTH) and interstitial-cell stimulating hormone (ICSH, metakentrin). In males, the maximum dose was 200 times (10.0 mgm.) the dose which caused a convincing testicular hypertrophy in comparison with uninjected control hypophysectomized males. The adrenals, seminal vesicles and ventral prostate were carefully weighed in all animals but microscopic examination of these tissues was limited to males receiving the largest doses and to their uninjected controls.

The preparation of follicle-stimulating hormone (FSH, thylakentrin) from sheep pituitaries has been described by Li, Simpson and Evans (1949). Dr. C. H. Li kindly gave us a sample of pure hormone (No. 1657D) as a dry solid which was used for all the comparisons with hog FSH. Hog FSH was prepared as follows. The initial extraction of whole hog pituitaries was carried out essentially as described by Chow *et al.* (1942). The final crude ammonium sulfate precipitate was dialyzed sulfate-free and dried in the frozen state. To an aqueous solution of powdered salt-free extract (2.5 gm. of extract per 100 ml. of water), one-half of a volume of saturated aqueous $(NH_4)_2SO_4$ solution (SAS) was added and the pH was adjusted to 7.35 with 1.0 N NaOH very slowly and with constant stirring. (More dilute NaOH solution or saturated $NaHCO_3$ solution has been used for pH adjustment without improvement in final products.) The precipitate which appeared contained crude metakentrin (ICSH or LH) and was removed by centrifugation. The supernatant fluid, containing other impurities as well as the bulk of the FSH, was adjusted to pH 4.8 and solid $(NH_4)_2SO_4$ was added to 0.5 saturation and dissolved at room temperature. The precipitate appearing (between 0.33 and 0.5 SAS) was likewise separated by centrifugation and set aside for reworking. Three-tenths of a volume of SAS were mixed with the supernatant raising the saturation of the solution with $(NH_4)_2SO_4$ from 0.5 to approximately 0.6. This precipitate, separated by centrifugation, contained the bulk of the FSH and was subjected to reprecipitation by dissolving in the minimum necessary volume of water, following which 1.5 volumes of SAS were added with stirring and the precipitate was again separated by centrifugation. This last step was repeated at least seven times although it is improbable that any improvement occurred after the fifth reprecipitation. The final precipitate was dialyzed sulfate-free and dried in the frozen state. The dried powder will be subsequently referred to as hog thylakentrin or FSH.

ELECTROPHORESIS

The electrophoretic studies were carried out in a Longsworth scanning apparatus. In the preliminary work experiments were conducted on preparations which were dialyzed against acetate, cacodyl-

late, or diethylbarbiturate buffers which covered a range of pH from 3.5 to 8. Porportions of material corresponding to the several electrophoretic components present were withdrawn from the cell at the end of the experiments for assay in order to determine which of them corresponded to the hormone.[2] From a plot of the mobility values for the active material against the corresponding values of pH, the iso-electric point was found to be near 4.8 which is close to the value, 4.5, estimated by Li (1949) for sheep FSH.

Later work made use of electrophoretic analysis to follow the extent of purification achieved by the various procedures which were employed, and most of these determinations were carried out at pH 7.8 and ionic strength 0.05 in a buffer composed of 0.01 M diethylbarbituric acid, 0.01 M of its sodium salt and 0.04 M sodium chloride. The best of the preparations we were able to obtain appeared to be between 80 and 85 per cent electrophoretically pure. The mobility of of the active mmaterial in this buffer was about -7×10^{-5} (cm./sec.)/(volt/cm..) The inactive material consisted of two electrophoretic components having mobilities of about -4.5×10^{-5} and -3×10^{-5} with more of the faster than of the slower one. However in less pure preparations containing 55–70 per cent of active substance these two impurities were present in nearly equal amounts.

An analysis of Li's sheep preparation in the same buffer (pH 7.8) carried out in our apparatus showed that it had about the same mobility as our active hog material and was essentially pure containing only a few per cent of a component with a mobility of -3.5 to -4.0×10^{-5}.

OTHER ATTEMPTS TO PURIFY HOG FSH

Ethanol Fractionation. In view of the success of Cohn and his collaborators in separating plasma proteins by ethanol fractionation under various conditions at low temperatures, a number of experiments was performed with hog pituitary extracts rich in FSH at -5 to $-7°$ C. which was the lowest possible temperature in the refrigerated room available. The crude or purified hormone was dissolved in acetate (pH 4.00 to pH 5.40) or barbital buffer (pH 8.43 to pH 8.60) at ionic strengths of 0.01 to 0.1. Ethanol, sometimes also containing buffer in the same ionic strength, was added slowly with constant stirring and fractions precipitating in the range of 5 to 50 per cent ethanol were separated by centrifugation, suspended in water and dried in the frozen state. No useful separation of pure hormone from impurity occurred.

[2] Kirkwood has developed an apparatus for protein fractionation by convection-electrophoresis (Kirkwood, 1940; Nielson and Kirkwood, 1946). Through the courtesy of Dr. Mary H. Loveless, we were able to use her Kirkwood apparatus in one of our last experiments for attempting to purify the hormone. A small but definite enrichment of the active material was obtained (from 75 per cent initial to 82 per cent final). However, this promising approach for purification has not been adequately tested.

After the publication of the method of Li, Simpson and Evans (1949), the behavior of purified hog FSH in strong ethanol solutions at a low temperature was examined. If the extract was dissolved in water, the bulk of the hormone was precipitated at an ethanol concentration of 40 per cent and there was little additional precipitate when enough ethanol was added to reach a concentration of 80 per cent. On the other hand, if the purified hormone was dissolved in 0.167 M K_2HPO_4 (pH 9.25), and absolute ethanol was added slowly at $-5°$ C. to a concentration of 40 per cent with a reduction of the molarity of the K_2HPO_4 to 0.1, the dissolved extract remained in solution. Nearly two-thirds of the purified hormone was precipitated when ethanol was then added to a concentration of 80 per cent. Therefore, hog FSH, like sheep FSH as described by Li, Simpson and Evans, is soluble in 40 per cent ethanol provided that there is also present 0.1 M K_2HPO_4. The purified hog hormone precipitated when the concentration of ethanol was raised from 40 to 80 per cent has been subsequently fractionated by the method of Li, Simpson and Evans. Whereas electrophoretically pure sheep FSH can thus be obtained, hog FSH contains about the same amount of impurity as the product obtained by the salt-fractionation method first described. It appears that sheep pituitary glands are more suitable starting material for FSH than hog glands.

Extraction of Lipids. It is conceivable that lipid or lipoprotein contaminated the best preparations of hog FSH. Accordingly, purified hormone was extracted in a deep-freeze unit by absolute acetone, or absolute ether or a mixture of one part of absolute ether and three parts of absolute alcohol. The extraction was carried out for 11 hours at $-29°$ C. and the suspending solvents were removed under vacuum at a low temperature. The product was not improved by this treatment and there was a definite loss of potency in the batches of purified hormone extracted with absolute ether or with the mixture of ether and ethanol.

Fractionation with Sodium Sulfate. Sodium sulfate, as a 40 per cent solution at 37° C., was also added to various concentrations as a precipitating agent. The bulk of the hog FSH was precipitated between the concentrations of 20 and 27.5 per cent Na_2SO_4. All efforts to improve the purity of the product by fractional precipitation between these limits failed.

Experiments with Nucleic Acid. The possibility that separation of hog FSH from impurities could be accomplished by reaction with nucleic acid was also explored. A solution of 0.5 per cent sodium nucleate derived from yeast (Schwarz Laboratories) was mixed with an equal volume of 2 per cent hog FSH extract (80 per cent pure) and the pH of the mixed solution, which was clear at a PH of 5.60 or higher, was lowered serially to pH 3.75. Separable precipitates could be removed for assay in the pH range of 5.05 to 4.00. There was no

appreciable separation of hormone from impurity. It was hoped that the latter might interact with the nucleic acid and could be removed by centrifugation.

Isoelectric Precipitation; Stability at Various Values of pH. The isoelectric point of hog FSH appears to be about pH 4.8. This is close to the pH of 0.6 saturated $(NH_4)_2SO_4$ (pH 4.6–4.7) at which hormone which is 80 to 85 per cent pure can be precipitated. Numerous attempts to secure crystalline or pure hormone near the isoelectric point by pH adjustment of solutions containing varying concentrations of hormone all failed. Isoelectric precipitation of pure hog FSH also could not be accomplished by mixing various concentrations of impure hormone with acetate buffer, pH 4.8, in different ionic strengths or by dialyzing such buffers into a rotating cellulose tube containing a solution of purified hog FSH.

TABLE 1. COMPARISON OF EFFECTS OF HOG AND SHEEP FOLLICLE-STIMULATING HORMONES IN HYPOPHYSECTOMIZED IMMATURE FEMALE RATS

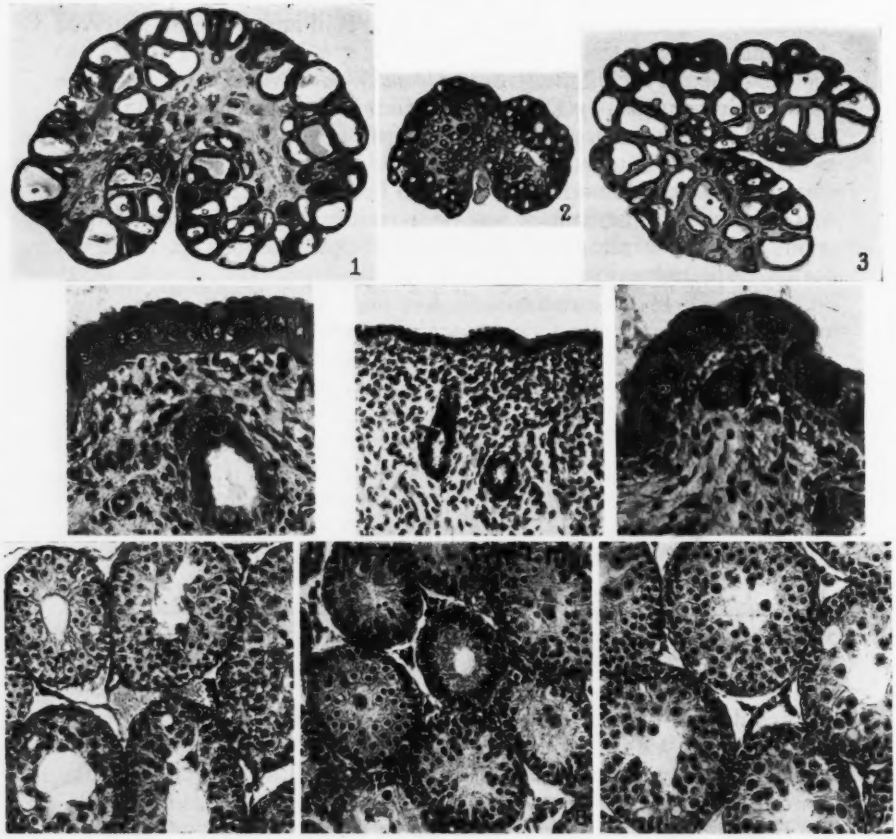
Preparation	Total dose	No. of rats	Ovaries	Uterus	Adrenals
	mg.		mg.	mg.	mg.
Hog 446	0.05*	5	8.7±1.42	15.0±1.65	10.5±0.81
Sheep 1657D	0.05*	6	8.5±1.07	13.6±1.85	8.9±0.85
Hog 446	0.1*	6	9.8±1.05	14.0±1.26	10.3±1.28
Sheep 1657D	0.1*	6	8.0±0.91	12.4±0.96	9.6±1.00
Hog 446	2.0†	3	32.3	85.3	8.0
Sheep 1657D	2.0†	2	45.2	94.9	8.3
None	0	6	9.1±1.22	13.5±1.69	9.4±1.31

The standard deviation is given with mean values.
* Total dose distributed over 3 days.
† Total dose distributed over 4 days.

Hog follicle-stimulating hormone is destroyed if kept at room temperature for 4 hours at pH 1.12 or lower. There is little destruction between pH 3.15 and pH 10.90 under these conditions. Stability is excellent at about pH 5.10.

COMPARISON OF BIOLOGICAL EFFECTS OF HOG AND SHEEP FOLLICLE-STIMULATING HORMONES

Gonadotrophic Effects in Female Rats. The comparison of the effects of FSH from the two species in hypophysectomized female rats was made according to the dosage schedule used by Li, Simpson and Evans (1949) in rats hypophysectomized at an age of 27 days. Injections were begun 7 days later. The smaller doses did not affect ovarian or uterine weights. The largest dose caused marked hypertrophy of both the ovaries and the uterus which was weighed after the removal of fluid. These results are summarized in table 1. The secretion of estrogen by the ovary of the hypophysectomized rat does



EXPLANATION OF FIGS. 1 TO 9
(All the tissues are from hypophysectomized immature rats.)

Fig. No.	Organ	Magnification	FSH Injected	
			Source	Total dose mg.
1	Ovary	×13.5	Sheep	2.0
2	Ovary	×13.5	—	0
3	Ovary	×13.5	Hog	2.0
4	Uterus	×230	Sheep	2.0
5	Uterus	×230	—	0
6	Uterus	×230	Hog	2.0
7	Testis	×165	Sheep	10.0
8	Testis	×165	—	0
9	Testis	×165	Hog	10.0

not occur after the administration of follicle-stimulating hormone alone but requires the additional injection of interstitial-cell stimulating hormone (Greep *et al.*, 1942). The association of uterine changes characteristic of estrus with microscopic evidence of the repair of the ovarian interstitial cells justifies the view that the preparations of FSH caused estrogen-secretion because of contamination with interstitial-cell stimulating hormone.

Histological examination of the ovaries and uteri revealed no difference in the effects of the sheep and hog FSH in hypophysectomized immature female rats. Total doses of 50 micrograms of hormone caused no recognizable microscopic changes. Beginning antrum formation was evident in the ovaries after doses of 100 micrograms. Very large doses of either preparation, i.e., 2.0 mgm., caused repair of the interstitial cells, thickening and luteinization of the theca with marked mitotic activity in the granulosa cells. After the two smaller doses there was no gross or microscopic alteration of the uterus. After the dose of 2.0 mgm. of either preparation there was evidence of marked secretion of estrogen as shown by uterine hypertrophy and hypertrophy and hyperplasia of the uterine epithelium. Representative photomicrographs are reproduced in figures 1 to 6.

Gonadotrophic Effects in Male Rats. A comparison of the potency of the sheep FSH and impure hog FSH as assayed in hypophysectomized male rats is summarized in table 2. When small doses were compared (0.05 or 0.1 mg.) the two preparations were indistinguishable. The smallest dose used (0.05 mgm.) caused a significant testicular hypertrophy. For example, the probability that the average testicular weight of the 12 rats receiving 0.05 mgm. of the hog prep-

TABLE 2. COMPARISON OF EFFECTS OF HOG AND SHEEP FOLLICLE-STIMULATING HORMONES IN HYPOPHYSECTOMIZED IMMATURE MALE RATS

Preparation	Total dose	No. of rats	Testes	Anterior prostate	Seminal vesicles	Adrenals
	mg.		mg.	mg.	mg.	mg.
Hog 446	0.05	12	145 ± 18.9	7.0 ± 1.74	5.1 ± 0.75	8.4 ± 1.02
Sheep 1657	0.05	12	146 ± 21.7	6.6 ± 1.15	5.0 ± 0.47	8.5 ± 1.32
Hog 446	0.1	12	173 ± 23.7	7.1 ± 1.20	5.4 ± 0.73	8.1 ± 1.17
Sheep 1657	0.1	12	170 ± 25.7	6.4 ± 0.89	5.2 ± 0.73	8.0 ± 0.93
None	0	12	124 ± 13.8	6.3 ± 0.60	5.2 ± 0.56	8.0 ± 0.95
Sheep 1657	10.0	4	379 ± 27.5	20.6 ± 1.40	6.5 ± 0.38	9.1 ± 1.40
Hog 446	10.0	4	348 ± 21.9	15.4 ± 4.05	4.8 ± 0.31	6.7 ± 2.16
Hog 291	10.0	3	374 ± 18.1	19.6 ± 4.34	5.3 ± 0.31	9.5 ± 0.32
Hog 291 Dig.*	10.0	4	315 ± 30.2	9.1 ± 1.22	4.5 ± 0.64	7.8 ± 0.61
Hog 291 Dig.*	20.0	4	328 ± 40.4	11.2 ± 1.41	4.9 ± 0.17	8.6 ± 1.15
Hog 291 Dig.*	0.1	5	128 ± 6.5	7.9 ± 2.60	4.5 ± 0.18	7.4 ± 0.86
None	0	7	117 ± 13.8	7.2 ± 1.23	4.1 ± 0.36	7.9 ± 0.77

The standard deviation is given for each mean value.
* Partial digestion by Merck trypsin. Dosages are in terms of original material.

aration could occur in an uninjected control group is less than 0.01. The qualitative effects of the hog and sheep hormones are brought out in the lower part of the table. Only a few animals could be used for each preparation since a dose two hundred times the threshold dose was employed. The fact that the sheep FSH produced as much hypertrophy of the anterior prostate as the hog FSH suggests that both preparations were still contaminated with metakentrin (ICSH or LH). Partial digestion of one of the hog preparations with impure trypsin originally obtained from Merck of Darmstadt abolished much but not all of the interstitial-cell stimulation as shown by the reduced hypertrophy of the anterior prostate. There was also a reduction of potency by partial digestion.

Identical effects as judged by histological examination were produced by either preparation in male hypophysectomized rats. The very large doses (10 mgm. or 200 times the minimal dose causing a significant hypertrophy of the testes) undoubtedly repaired both the germinal epithelium and the interstitial cells. Numerous primary spermatocytes appeared in the tubules. Although a morphological change in the interstitial cells was not demonstrated, the androgen stimulation of the ventral prostate was striking. In the latter the epithelium in many areas was columnar and undergoing hyperplasia and there was much accumulated secretion in the acini. It appears that neither preparation was biologically pure and that both, in equally large doses, caused interstitial-cell stimulation leading to estrogen-secretion by the ovaries and androgen-secretion by the testes. Figures 7 to 9 illustrate the histological changes in the testes.

Immunological Comparison of Follicle-Stimulating Hormone from Sheep and Hogs. This comparison was limited to the precipitin reaction owing to the small amount of pure sheep FSH available. Fortunately, both proteins were satisfactorily antigenic.

Antisera were produced in rabbits by the intravenous injection of alum-treated protein. A solution of either protein (10 mg. per ml.) was mixed with 0.1 volume of 1 per cent potassium alum and the pH of the mixture was adjusted to 5.50–5.75. The colloidal suspension was distributed in vials in volumes sufficient for daily requirements and was kept frozen until use. On thawing at the time of injection, the suspension remained uniformly colloidal. Healthy adult male rabbits each received the equivalent of 5 mg. of hormone intravenously the same three days each week for two weeks or longer. Satisfactory titers of serum precipitins were obtained from either hormone after injection for two weeks. Two rabbits received hog FSH for a total of five weeks with bleedings after two weeks and later. There was sufficient sheep FSH for immunizing only one rabbit for three and a half weeks but the serum of this rabbit fortunately had a high precipitin titer after two weeks.

For the precipitin tests 0.3 ml. of serum was mixed with an equal

volume of saline or diluted antigen (hog or sheep FSH). The tubes were read at room temperature the same day and again after 24 and 48 hours in the refrigerator. Final readings were made after centrifugation. A typical experiment comparing the reaction of a serum with both the homologous and the heterologous antigens is summarized in table 3. It is clear that precipitating antibodies were present in each serum but that the reaction was specific for the antigen used for immunization.

In another experiment, 2.0 ml. samples of hog FSH antiserm were mixed with increasing quantities of sheep FSH (0.01 mg. N

TABLE 3. THE PRECIPITIN REACTION OF SERA OF RABBITS IMMUNIZED WITH HOG OR SHEEP FOLLICLE-STIMULATING HORMONE

Source of FSH used as antigen	Test hormone	Final dilution of test hormone								
		1:2,000	1:6,000	1:12,000	1:18,000	1:30,000	1:54,000	1:162,000	1:486,000	1:1,458,000
Hog anterior pituitary	Hog FSH	+	+++	++++	++++	+++	++	+	−	−
Hog anterior pituitary	Sheep FSH	−	−	−	−	−	−	−	−	−
Sheep anterior pituitary	Hog FSH	−	−	−	−	−	−	−	−	−
Sheep anterior pituitary	Sheep FSH	+	++++	++++	++++	+++	++	+	±	−

followed by 0.02 mg. N and 0.07 mg. N at intervals of 7 days). Throughout this period the tubes were refrigerated. No precipitate appeared and the precipitin reaction of the serum to hog FSH in comparison with the same serum to which saline had been added was not impaired. Therefore, even large amounts of sheep FSH neither led to a precipitin reaction with hog FSH antiserum nor inhibited the precipitin reaction with the homologous antigen (hog FSH). All the results justify the conclusion that the hog and sheep follicle-stimulating hormones, being immunologically distinct, are therefore chemically different proteins.

DISCUSSION

As was mentioned in the introduction, metakentrins (ICSH or LH) from hog and sheep glands appear to be different proteins with widely different molecular weights (hog: 100,000; sheep: 40,000) and isoelectric points (hog: pH 7.45; sheep: pH 4.60). They also are completely distinct antigenically. From the evidence here reported it may be concluded that the follicle-stimulating hormones (thylakentrins) from these two species are also different proteins since they show individual immunological specificity. On the other hand, the isoelectric points for these two substances are quite close together (hog: pH 4.8; sheep: pH 4.5). It was not possible to compare the molecular weights in view of the fact that the preparation obtained from hog glands was not considered sufficiently pure for this purpose. The endocrine potency and effects of the two hormone proteins were identical so far

as could be judged from assays in hypophysectomized male and female immature rats. Both contained traces of metakentrin (ICSH or LH).

A comparison of the lactogenic hormone from beef and sheep glands appeared to demonstrate no immunological difference (Bischoff and Lyons, 1939). Although these authors did not prove that the hormones from the two species are identical, they showed that the immunological relationship is very close. Yet these substances showed a difference in tyrosine content and solubility characteristics (Li, Lyons, and Evans, 1940 (a, b); White, Bonsnes and Long, 1942). On the other hand, adrenal cortical stimulating hormones from hogs and sheep appear to be chemically alike (White, 1946). Whether the corticotrophins from hog and sheep glands are immunologically similar has not been determined. Experiments with thyroglobulins as antigens have demonstrated a closer species-relationship between the ox and the sheep than between the hog and the sheep (Kabat and Mayer, 1948).

It would appear, therefore, that two isobiological substances may differ or be similar in chemical and physico-chemical properties. Also, two substances which are isobiological in one respect (hormone) may or may not be isobiological in another (antigen). It is true that as yet no clearcut case, including insulin, has been found in which corresponding hormones from two different species are indentical in every one of the respects mentioned above. However, one cannot assume that such an identity may not be demonstrated for a particular hormone in the future.

SUMMARY

Several procedures have been investigated for the isolation of follicle stimulating hormone (FSH, thylakentrin) from hog pituitary glands using electrophoretic analysis as a guide. The most satisfactory employed salt fractionation with ammonium sulfate and yielded material which had a maximum purity of 80 to 85 per cent hormone.

A comparison with the highly purified sheep FSH of Li, Simpson and Evans revealed (a) no difference in potency or hormonal purity by assay in hypophysectomized rats, (b) practical identity of isoelectric points (pH 4.5 and 4.8), and (c) an unquestionable immunological difference.

The significance of these findings is discussed.

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THE ROLE OF HORMONES IN ADIPOSE GLYCOGEN SYNTHESIS IN THE RAT. INSULIN AND THE HYPERGLYCEMIC FACTOR OF THE PANCREAS

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THE STUDY of the role of hormones in fat metabolism has been handicapped by a paucity of methods suitable for investigating this problem. With the increasing evidence, established largely by the isotope technique, that adipose tissue is more dynamically active than had been once thought and may be a site of conversion of carbohydrate to fat, the studies of Wertheimer and his colleagues (Wertheimer, 1927, Tuerkischer and Wertheimer, 1942, Wertheimer, 1945, Tuerkischer and Wertheimer, 1946, Wertheimer and Shapiro, 1948) on adipose glycogen assume increased importance. In a series of papers going back to 1927 these investigators have described the various factors involved in glycogen accumulation in adipose tissue. They have adduced evidence to support the view that glycogen deposition in adipose tissue may be correlated with liponeogenesis from carbohydrate in this tissue. The evidence may be summarized as follows. 1. Conditions favoring glycogen deposition are generally the same as those enhancing fat synthesis. Thus, high glycogen levels were found in rats refed after starvation, rats trained to consume their entire ration in one hour (Tepperman, Brobeck and Long, 1945), rats fed and starved on alternate days, after excessive carbohydrate intake, and after insulin injection in either the fed or fasted state. 2. Glycogen accumulation precedes fat deposition and comes to an end with completion of fat deposition. 3. Glycogen levels are low in conditions characterized by impaired lipogenesis, i.e., diabetes mellitus (Stetten and Boxer, 1944) and thiamine deficiency (Boxer and Stetten, 1944). 4. The respiratory quotient of adipose tissue containing glycogen exceeds 1.0 in vitro (Mirski, 1942). Additional evidence, both indirect and direct, that adipose tissue is indeed a site of conversion of carbohydrate to fat is found in the observations of Tepperman *et al.* (1943) and Masoro *et al.* (1948). The former have reported that the R. Q. of eviscerated rats previously trained to consume their daily ration in 1 hour exceeds 1.00 after glucose and insulin, while the latter isolated

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C^{14} labeled palmitic acid from eviscerated rats which had received C^{14} labeled glucose.

On the premise that adipose glycogen may bear some interpretable relation to the rate of liponeogenesis from carbohydrate a series of studies have been instituted on the relation of hormones to adipose glycogen deposition. Because of its high metabolic activity and greater propensity to synthesize glycogen rapidly, the dorsal interscapular brown fat of the rat has been used in most of these experiments. Brown fat is more highly saturated than white fat, which may be indicative of the fact that more of it arises from conversion of carbohydrate to fat. Because it is so well developed in hibernating animals and relatively underdeveloped or absent in other species, some question may be raised as to whether the dorsal interscapular fat may not have some specialized function, and hence metabolic changes observed in it may not be representative of adipose tissue in general. However, since histologically and chemically it does consist of fat and little else, hormonal effects on this tissue are still meaningful with regard to the general problem of whether hormones do influence fat metabolism. Some observations have also been made on white fat, which differs considerably from brown fat in its rate and magnitude of glycogen synthesis, but these will be the subject of a later communication.

The present report is concerned with the effects of insulin and the hyperglycemic factor of the pancreas on fat glycogen and in general confirms earlier reports that the former hormone stimulates glycogen synthesis in fat. By the use of measured tube feeding it has been possible to establish more definite quantitative relationships in the glycogen response to glucose and insulin.

METHODS

Male albino rats of the Vanderbilt strain were used. For each experiment animals of closely comparable weight were chosen. The rats were fasted 48 hours before experimental procedures were begun. Prior to fasting the rats were fed a diet of commercial chow, supplemented with liver pudding and lettuce twice a week (Engel, Schiller and Pentz, 1949). At the end of the experiments the animals were anesthetized with nembutal and the dorsal brown fat removed and dissected free of the surrounding white fat and weighed on a torsion balance. Samples weighing 100–400 mg. were transferred to 15 ml. centrifuge tubes containing 2 ml. of hot 30% KOH and digested on a steam bath for 20 minutes. The glycogen was precipitated by the addition of 3 ml. of 95% ethyl alcohol, the tube being returned to the steam bath and heated until the alcohol boiled. After standing overnight in the refrigerator the tubes were centrifuged and the supernatant discarded. In early experiments the precipitate was then hydrolyzed with 1 $N \cdot H_2SO_4$ as in the Good, Kramer and Somogyi procedure (1933), the glucose being determined by the photometric method of Somogyi (1945). Not uncommonly, however, it was found that sufficient fat remained with the glycogen to result in cloudy solutions which were unsatisfactory for the final photometric analysis. For this reason the procedure was modified to include

washing the glycogen precipitate twice with hot ethyl alcohol before hydrolysis. By this technique the fat is completely removed. Liver glycogen was determined by the method of Good, Kramer and Somogyi (1933); blood sugar by the method of Somogyi (1945).

In some experiments the rats were kept after removal of the interscapular fat. These animals survived and grew, failing to confirm the report of Vignes (1913) that rats waste away and die after removal of this tissue.

TABLE 1. EFFECT OF INSULIN IN 48 HOUR FASTED AND GLUCOSE-FED RATS

	Average body weight in grams	Adipose glycogen grams/100 gm. fat	p§	Liver glycogen grams/100 gm. liver	p	Blood sugar mg/100 ml.	p
Control	114	0.052±0.007 (20)		0.728±0.077 (14)		92±3.0 (31)	
Insulin* 0.5 Unit	121	0.180±0.048 (8)	<.02	0.450±0.110 (10)	<.05	79±5.7 (10)	<.05
Glucose†	113	0.690±0.115 (27) p‖<.02	<.01	3.10 ±0.309 (16) p<.05	<.01	229±8.4 (22) p>0.5	<.01
Glucose 2 Units Insulin‡	111	1.127±0.141 (12)	<.01	2.07 ±0.307 (5)	<.01	230±16 (6)	<.01

Results expressed as mean ± standard error. Number in parentheses refers to number of animals.
* 0.17 unit insulin at 0, 1, and 2 hours, sacrificed at 8 hours.
† 2 ml. 50 per cent glucose p.o at 3 and 5 hours, sacrificed at 8 hours.
‡ 2 units of insulin at 0, 2 ml. 50 per cent glucose at 3 and 5 hours, sacrificed at 8 hours.
§ Significance compared to fasted controls.
‖ Significance between glucose and glucose and insulin groups.

RESULTS

As seen in Table 1, intraperitoneal administration of 0.5 unit of Squibb's insulin in divided doses at 0, 1 and 2 hours was followed by a significant increase in fat glycogen measured 8 hours later in the 48 hour fasted rat. The liver glycogen and blood sugar levels of the insulin-treated animals were lower than their controls, but not highly significantly so ($p<.05$). Administration of 1 gram of glucose as 2 ml. of 50% glucose by stomach tube at 3 and 5 hours was followed by significant increases in the fat and liver glycogen and blood sugar at 8 hours compared to the untreated and insulin treated fasted controls. When 2 units of insulin were given to the glucose fed animals, adipose glycogen levels were significantly higher than the controls and the liver glycogen lower ($p<.05$).

A series of experiments were performed to determine the roles of the time and dose of insulin in the adipose glycogen response. Using a single dose of glucose (1 ml. of 50% glucose/100 gms.) and a sample of insulin (Lilly) highly purified and free of hyperglycemic factor (1 unit/100 gms. body weight), the glycogen content of the dorsal fat was determined in groups of rats at 2 hour intervals up to 10 hours. As seen in Fig. 1, the maximum increase in glycogen took place by 4 hours. Thereafter glycogen values declined, but were still elevated at 10 hours. In these same experiments no significant changes in mesenteric, groin or perirenal fat glycogen took place, indicating that these tissues are much less reactive than the dorsal brown fat.

Four groups of rats were given 1 ml. of glucose per 100 gms. by stomach tube and 0, 0.5, 1.0 and 2.0 units of Lilly's insulin/100 grams

body weight intraperitoneally. Four hours later at the time of maximal glycogen formation they were sacrificed. When the resulting fat glycogen values were plotted against the dose of insulin on semi log paper a straight line relationship was obtained (Figure 2). Thus the adipose glycogen enhancing effect of insulin conforms to the usual dose-response relationship characteristic of biological phenomena in general.

Most commercial samples of insulin contain variable amounts of an hyperglycemic factor. The availability of a partially purified sample of the hyperglycemic factor from the pancreas (HGF Lilly E 1433) enabled us to test directly whether this material was involved

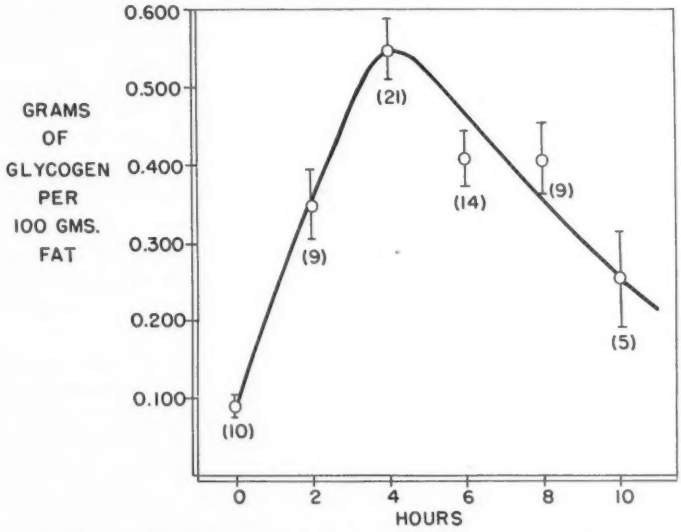

FIG. 1. Changes in the interscapular adipose glycogen content of 48 hour fasted rats during 10 hours after the administration by stomach tube of 500 mg. of glucose/100 grams body weight and 1 unit of insulin/100 grams intraperitoneally.

in the adipose glycogen response to insulin. The HGF was suspended in isotonic saline and brought into solution by the addition of a few drops of 0.1 N HCL. Four groups of 7 rats each were given 1 ml. of 50% glucose per 100 grams body weight by stomach tube after a 48 hour fast. Two hours later all were given light nembutal anesthesia and they were sacrificed 4 hours after glucose ingestion when glycogen synthesis would be maximal (Fig. 1). Group 1 received 0.2 ml. of normal saline intravenously at 2 hours. The second group received intraperitoneally 1 unit of insulin free of HGF per 100 grams body weight with the glucose and 0.2 ml. of normal saline intravenously at 2 hours; the third 0.1 mg. HGF in 0.2 ml. saline intravenously at 2 hours; and the fourth 1 unit of insulin per 100 grams with the glucose and 0.1 mg. of HGF at 2 hours. 0.5 mg. of this preparation

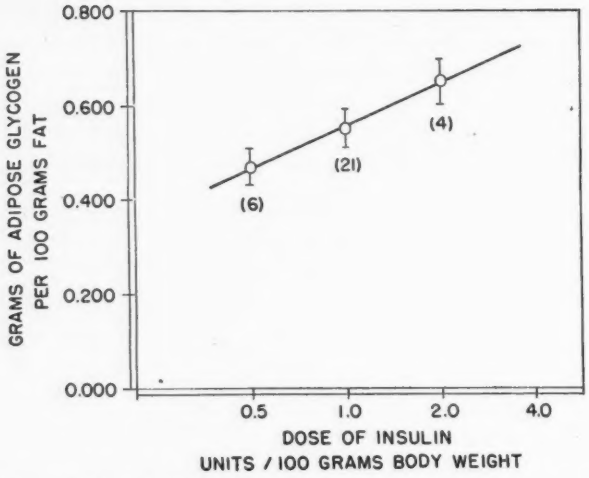

FIG. 2. Effects of increasing doses of insulin on the concentrations of glycogen in interscapular fat of the 48 hour fasted rat given 500 mg. of glucose per 100 grams.

of HGF produced a maximal blood sugar rise in a cat (Campbell, 1950). In the rat 0.1 mg. of HGF E 1433 caused a maximal increase in blood sugar in 15 minutes (Engel, 1950). The results are seen in Table 2.

Insulin produced its usual effect, increasing fat glycogen and decreasing liver glycogen and blood sugar. The HGF, on the other hand, had no influence on the fat glycogen, but lowered the liver glycogen and the blood sugar when measured 2 hours after injection. This latter finding suggested that this preparation of HGF was not free of insulin. HGF did not alter the response to insulin with respect to fat and liver glycogen but the final blood sugar was a little higher. It might be noted, incidentally, that the adipose glyocgen levels in the glucose and glucose-insulin treated rats in this series of animals

TABLE 2. EFFECTS OF INSULIN AND HYPERGLYCEMIC FACTOR (i.v.) IN 48 HOUR FASTED GLUCOSE-FED RATS

	No. of rats	Average body weight in grams	Adipose glycogen grams/100 gm. fat	p	Liver glycogen grams/100 gm. liver	p	Blood sugar mg/100 ml.	p
Glucose i.p. + Saline i.v.	7	125	0.168±0.034		2.89±0.210		120±8.2	
Glucose + Lilly's Insulin i.p. + Saline i.v.	6	137	0.469±0.094	<.01	0.50±0.130	<.01	40±5.9	<.01
Glucose i.p. + 0.1 mg. HGF (i.v.)	7	142	0.159±0.007	>0.1	1.22±0.165	<.01	90±6.8	<.02
Glucose + Lilly's Insulin i.p. + 0.1 mg. HGF i.v.	7	131	0.433±0.058	<.01	0.62±0.273	<.01	47±5.3	<.01

50% glucose, 1 ml./100 gm. by stomach tube at 0 time
Insulin, 1 unit/100 gm. intraperitoneally at 0 time
Saline 0.2 ml. and HGF 0.1 mg. in 0.2 ml. saline intravenously at 2 hours
} Sacrificed at 4 hours.

which received nembutal 2 hours before sacrifice were somewhat lower than usually found in animals treated identically but for the nembutal (Table 5 and Table 3 of Scott and Engel, 1950). This suggests that nembutal might have an inhibitory effect on fat glycogen synthesis.

Since HGF is ineffectual when given intraperitoneally (Campbell, 1950), it was possible to make a crude estimate of the amount of insulin in the HGF (Sample E1433) used in these experiments by comparing its effects to a known sample of insulin also given intraperitoneally. The results are seen in Table 3. The animals were treated exactly as in the previous experiment except that 1 mg. of HGF in 1 ml. of saline was injected intraperitoneally per 100 grams

TABLE 3. EFFECTS OF INSULIN AND HYPERGLYCEMIC FACTOR IN 48 HOUR FASTED GLUCOSE-FED RATS

	No. of rats	Average body weight in grams	Adipose glycogen grams/100 gm. fat	p	Liver glycogen grams/100 gm. liver	p	Blood sugar mg./100 ml.	p
Glucose	7	123	0.370±0.060		2.47±0.115		150±7.1	
Glucose+Lilly's Insulin	7	114	0.652±0.099	<.05	0.37±0.094	<.01	41±3.9	<.01
Glucose+HGF	7	123	0.566±0.074	<.05	0.98±0.241	<.01	71±9.2	<.01
Glucose+Lilly's Insulin+HGF	7	120	0.781±0.144	<.02	0.06±0.002	<.01	29±1.7	<.01

50% Glucose, 1 ml./100 gm. by stomach tube at 0 time
Insulin, 1 unit/100 gm. intraperitoneally at 0 time
HGF, 1 mg./100 gm. intraperitoneally at 2 hours
} Sacrificed at 4 hours.

into unanesthetized rats of groups 3 and 4. This amount of HGF resulted in an increase in fat glycogen of the same order as 1 unit of insulin, but a somewhat lesser fall in liver glycogen and blood sugar. When given with the insulin, the increase in fat glycogen was slightly greater, but not significantly so in this small series, while the liver glycogen and blood sugars were significantly lower. These data suggest that each milligram of HGF E1433 is contaminated by about 1 unit of insulin. The lack of effect of 0.1 mg. of HGF E1433 on fat glycogen in the previous experiment is therefore understandable since doses of the order of 0.1 unit of insulin have not resulted in measurable changes in the fat glycogen of the glucose fed rat.

DISCUSSION

These results confirm in general earlier reports (Wertheimer and Shapiro 1948, Fawcett 1948) that refeeding after a fast results in a significant increase in the glycogen content of interscapular adipose tissue and that this increase is augmented by insulin. In most of the previous studies ad libitum feeding was allowed so that it was not possible to obtain any concept of the quantitative relations between carbohydrate intake and glycogen synthesis. Fawcett (1948) tube fed his rats, but measured glycogen qualitatively by a histochemical technique. In the present study glucose was always administered by

stomach tube. Examination of the data in the different tables and figures shows, as might be expected, that the glycogen response increases as the dose of glucose is increased, but also increases as the dose of insulin is raised, the glucose intake being kept constant. The finding of a linear relation between the logarithm of the dose of insulin and the level of glycogen is most significant as regards the biological significance of the adipose glycogen response to insulin. It suggests that the increase in adipose glycogen represents a basic action of insulin. Since Masoro *et al.* (1949) have shown that lipogenesis from carbohydrate takes place in the absence of the viscera and presumably in the adipose tissue and Tepperman, Brobeck and Long (1943) have presented evidence that insulin promotes this conversion of carbohydrate to fat in the liverless rat, these quantitative data would tend to support the hypothesis of Wertheimer that glycogen may be a step in the conversion of carbohydrate to fat in adipose tissue. Stetten and Klein (1946) have reported that insulin will promote such a transformation in the liver of the normal animal. The failure of the diabetic rat to show glycogen in adipose tissue (Tuerkischer and Wertheimer, 1946) and the deficit in lipogenesis from carbohydrate in this same preparation (Stetten and Boxer, 1944) are further evidence for this interpretation.

On the basis of existing data it is not possible to draw any inferences concerning the actual magnitude of conversion of glucose to glycogen in adipose tissue in response to insulin. The total increase in glycogen in interscapular fat after insulin and 500 milligrams of glucose amounts to only about 1–2 milligrams more than after glucose alone, while in white fat which represents the major part of the fat depots no increase was measurable under these experimental conditions. Tuerkischer and Wertheimer (1946), on the other hand, with ad libitum high carbohydrate feeding and protamine zinc insulin found substantially greater increases in glycogen in both brown and white fat than reported here. This has been confirmed for white fat by Renold *et al.* (1949). Since at any moment the concentration of glycogen presumably represents a balance between synthesis and conversion to fat and each of these processes in turn probably will be dependent on the supply of carbohydrate and on the rate of fat catabolism respectively, it is clear that the level of glycogen cannot be interpreted in terms of the actual rate of conversion of carbohydrate to fat. Any experimental procedure which modifies the fat glycogen concentration will have to be evaluated in terms of the various factors involved in glycogen synthesis and degradation. In the case of insulin and pancreatectomy the interpretation is facilitated by the existing isotope data.

There is no evidence from the results reported here that the so-called hyperglycemic factor of the pancreas is in any way concerned with the observed phenomena in fat glycogen after insulin. Even

without the data presented here, this interpretation should be self evident from the fact that HGF is ineffectual when given by any route other than the intravenous one, whereas insulin is effective when given intraperitoneally.

In all the experiments reported here insulin injection regularly resulted in lower liver glycogen values than in controls similarly treated but not receiving insulin, but in all cases hypoglycemia was induced. It would appear that under the influence of insulin glucose is transferred from liver to muscle and adipose tissue.

SUMMARY

Insulin increases the glycogen content of interscapular adipose tissue of the 48 hour fasted rat both in the fasting state and after glucose administration.

After a single dose of glucose and insulin the increase in adipose glycogen is considerable by two hours, maximal at 4 hours and still demonstrable at 10 hours.

A linear relationship exists between the increase in glycogen and the logarithm of the dose of insulin.

There was no evidence that the hyperglycemic factor of the pancreas is concerned with adipose glycogen synthesis.

The significance of these findings with respect to conversion of carbohydrate to fat is considered.

We are indebted to Dr. E. D. Campbell of the Lilly Research Laboratories for a generous supply of insulin free of hyperglycemic factor and of a preparation of partially purified hyperglycemic factor of the pancreas, and to Rosalie Green and Mildred Engel for technical assistance.

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THE ROLE OF HORMONES IN ADIPOSE GLYCOGEN SYNTHESIS IN THE RAT. ANTERIOR PITUITARY GROWTH HORMONE

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ANTERIOR pituitary growth hormone exerts an influence on fat and carbohydrate metabolism in addition to its well known effect of promoting nitrogen storage and growth (Li and Evans, 1948). In the fasting animal, ketosis and an accumulation of fat in the liver occur rapidly after the injection of purified growth hormone (Bennett *et al.*, 1948, Szego and White, 1949). The latter authors showed that the adrenal is not essential for this response. In the fasting normal, hypophysectomized or adrenalectomized rat crude anterior pituitary extract (Harrison and Long, 1940) and purified growth hormone (Milman and Russell, 1949) both produce a prolonged depression of the blood sugar. These latter observations suggest that some of the effects of growth hormone may be mediated in part by insulin. Such a viewpoint was first proposed by Mirsky in 1939 and further developed by Young (1939, 1941, 1944, 1945) and by Gaebler and Galbraith (1941) and Gaebler and Robinson (1942). Young (1945) has suggested that part of the insulin response after growth hormone treatment may be concerned with an increased conversion of carbohydrate to fat to replenish depleted fat stores. However, the more recent demonstrations that purified growth hormone is diabetogenic in the dog, cat and rat (Houssay and Anderson, 1949) and raises the blood sugar of the alloxan diabetic rat (Milman and Russell, 1949), and the finding that the characteristic in vitro effect of insulin on glycogen synthesis in the rat diaphragm is inhibited if the animals are pretreated with growth hormone (Li, Kalman and Evans, 1949) suggest that any role insulin may be playing in the observed phenomena after growth hormone must be secondary to the metabolic effects of the latter hormone.

The purpose of this study was to determine whether any of the effects of purified anterior pituitary growth hormone on carbohydrate and fat metabolism might be reflected in changes in the levels of adi-

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pose tissue glycogen. In a previous report (Engel and Scott, 1950) the findings of Tuerkischer and Wertheimer (1946) and of Fawcett (1948) that insulin stimulated adipose glycogen synthesis were confirmed and extended. Since the effects of this hormone on fat glycogen were so clear cut, it seemed reasonable to assume that growth hormone might have a similar effect if its injection stimulated insulin production by a direct action on the pancreas. Contrariwise, since adipose glycogen synthesis is inhibited in diabetes (Tuerkischer and Wertheimer, 1946) and, as indicated above, growth hormone is diabetogenic and counteracts the action of insulin under certain circumstances, an inhibitory action of growth hormone on adipose glycogen after insulin might be anticipated. Neither effect was demonstrated in these experiments.

METHODS

These were the same as in the previous report. The growth hormone preparations used in these experiments were supplied by Dr. Alfred Wilhelmi and were highly purified (Fishman, Wilhelmi and Russell). The hormone was dissolved in 0.9% saline with the aid of a few drops of 0.1 N NaOH and prepared freshly immediately before use.

RESULTS

In the 48 hour-fasted rat injection of 1 milligram of growth hormone in divided doses 3 hours apart resulted in a significant depression of the blood sugar 5 hours after the last injection, but no change in either the dorsal fat glycogen or liver glycogen levels (Table 1). In another experiment 60–70 gram rats were given 2 milligrams of growth hormone in divided doses 3 hours apart and the animals sacrificed 9 hours after the last dose of growth hormone. Under these circumstances hypoglycemia was no longer present and again there was no difference in the fat glycogen levels. The liver glycogens of the growth hormone treated rats were lower than the controls. There is no ready explanation for this finding.

TABLE 1. EFFECT OF GROWTH HORMONE IN 48 HOUR-FASTED RATS

	Average body weight in grams	Adipose glycogen grams/100 gms. fat	p	Liver glycogen grams/100 gm. liver	p	Blood sugar mg./100 ml.	p
Control*	114	0.052 ± 0.007 (20)		0.728 ± 0.077 (14)		92 ± 3.0 (31)	
0.5 mg. G.H. at 0 & 3 hrs.*	122	0.059 ± 0.011 (13)	> .5	0.943 ± 0.080 (14)	> .1	67 ± 3.1 (14)	< .01
Control†	69	0.102 ± 0.011 (11)		1.020 ± 0.260 (9)		104 ± 6.7 (10)	
1.0 mg. G.H.†	67	0.087 ± 0.008 (8)	> .1	0.414 ± 0.065 (8)	< .02	107 ± 4.1 (8)	> .1

* Sacrificed at 8 hours.
† Sacrificed at 12 hours.

As seen in Table 2, when growth hormone was given to glucose fed rats no significant changes in either adipose glycogen, liver glycogen or blood sugar resulted in 8 hours, regardless of whether imma-

TABLE 2. EFFECT OF GROWTH HORMONE ON 48 HOUR-FASTED GLUCOSE FED RATS

	Average body weight in grams	Adipose glycogen grams/100 gm. fat	p	Liver glycogen grams/100 gm. liver	p	Blood sugar mg./100 ml.	p
1 gm. Glucose at 3 & 5 hrs.	113	0.690±0.115 (27)		3.10±0.309 (16)		229± 8.4 (22)	
0.5 mg. G.H. at 0. & 3 hrs. 1 gm. Glucose at 3 & 5 hrs.	114	0.863±0.089 (19)	<.3	2.95±0.129 (18)	>.5	232±13.9 (20)	>.5
2 gms. Glucose at 3 & 5 hrs.	332	0.471±0.149 (4)		1.68±0.265 (4)		210±12.3 (4)	
1.5 mg. G.H. at 0 & 3 hrs. 2 gm. Glucose at 3 & 5 hrs.	299	0.408±0.201 (3)	>.5	1.31±0.120 (3)	<.3	250±32.6 (3)	<.4
1 gm Glucose at 0 hour	98	0.285±0.060 (3)		3.03±0.503 (3)		96±0 (3)	
1 mg. G.H. & 1 gm. Glucose at 0 hour	90	0.300±0.110 (4)	>.5	2.14±0.301 (4)	<.1	107±4.6 (4)	<.2

All rats sacrificed at 8 hours.

ture or mature rats were used or whether the glucose was given with or after the growth hormone.

And finally, the absence of an inhibitory effect of growth hormone on the insulin stimulation of fat glycogen or depression of liver glycogen is shown in Table 3. Four groups of rats were fasted for 48 hours. All were given 1 milliliter of 50 per cent glucose per 100 grams by stomach tube. The first group received nothing else, the second 1 unit of insulin per 100 grams, the third 1 mg. of growth hormone per 100 grams and the fourth 1 unit of insulin and 1 mg. of growth hormone per 100 grams. All were sacrificed 4 hours later at the time when the adipose glycogen response is maximal (Engel and Scott, 1950). Note that insulin produced its usual effect on the adipose and liver glycogen and blood sugar and that this response was not altered by the simultaneous administration of growth hormone. The growth hormone had no significant effect on the adipose and liver glycogen or blood sugar. It is of some interest to point out the close correspondence between the values for fat and liver glycogen and blood sugar in this series of rats treated with glucose and glucose plus insulin and those in Table IV and Figs. 1 and 2 of the previous paper (Engel and Scott, 1950) which were from animals identically treated.

TABLE 3. EFFECTS OF INSULIN AND GROWTH HORMONE IN 48 HOUR-FASTED GLUCOSE FED RATS

	No. of rats	Average body weight in grams	Adipose glycogen grams/100 gm. fat	p	Liver glycogen grams/100 gm. liver	p	Blood sugar mg./100 ml.	p
0.5 gm. Glucose per 100 gms.	13	112	0.349±0.048		2.01±0.180		138±5.2	
0.5 gm. Glucose & 1 unit Insulin per 100 gms.	6	120	0.501±0.030	<.02	0.22±0.053	<.01	52±2.1	<.01
0.5 gm. Glucose & 1 mg. G.H. per 100 gms.	6	117	0.321±0.022 p*<.01	>.5	2.47±0.304 p*<.01	>.5	138±7.2 p*<.01	>.5
0.5 gm. Glucose & 1 mg. G.H. & 1 unit Insulin per 100 gms.	6	123	0.520±0.057	<.05	0.35±0.071	<.01	50±8.1	<.01

All rats sacrificed at 4 hours.
* Significance between Glucose and Growth Hormone vs. Glucose and Insulin and Growth Hormone.

DISCUSSION

The failure of anterior pituitary growth hormone to alter fat glycogen in the face of a fall in blood sugar argues against the hypoglycemia after growth hormone being solely an action of insulin unless anterior pituitary growth hormone directly inhibits the insulin effect on fat glycogen. This did not prove to be the case in these experiments, although such an inhibitory effect has been noted on glycogen synthesis in vitro in diaphragms of rats treated with growth hormone (Li *et al.*, 1949). In the glucose-fed rat, too, no effect of growth hormone on adipose glycogen was demonstrable. In the present state of our knowledge concerning adipose glycogen it is not possible to draw any conclusions from these negative results with anterior pituitary growth hormone as regards an effect or lack of effect of growth hormone on lipogenesis from carbohydrate.

SUMMARY

Anterior pituitary growth hormone has no effect on the concentration of glycogen in the interscapular adipose tissue of the 48 hour fasted rat given glucose, glucose and insulin or in the fasting state, even though its injection is followed by hypoglycemia during the fasting state. This is taken as evidence against the view that anterior pituitary growth hormone exerts an insulinotrophic effect on the pancreas.

We are indebted to Dr. Alfred Wilhelmi of the Department of Physiological Chemistry, Yale University for generous supplies of purified growth hormone and to Mrs. Rosalie Green and Mildred Engel for technical assistance.

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NOTES AND COMMENTS

THE EFFECT OF SHAW-GREEP DIET #100 ON THE ANEMIA WHICH IS INDUCED BY HYPOPHYSECTOMY IN THE ADULT FEMALE RAT[1]

Diet is important in work with the hypophysectomized animal. Recently Shaw and Greep (1949) made a thorough study of the effects of three prepared diets on the length of survival of rats after removal of the hypophysis. They found that hypophysectomized rats on these diets not only survived for longer periods of time after surgery but also increased in size and weight.

It seemed of interest to determine the effects of one of these diets, #100, on the anemia which has repeatedly been shown to be induced by hypophysectomy in the rat.

Of the 48 adult female rats (Long-Evans strain—6 to 7 months of age) used in this experiment, 7 served as controls, 30 were hypophysectomized and placed on the regular pellet diet,[2] and 11 were hypophysectomized and given the prepared diet.[3] Water was given ad libitum. Blood was studied before surgery and every 10 days after hypophysectomy for 50 days. The methods used were the same as have been reported from this laboratory repeatedly (Crafts, 1949).

Table 1 shows the results obtained. While the control rats showed a 10% increase in weight over the 50 days of observation (186 to 208 gm.), the hypophysectomized rats on the regular pellet diet decreased from an average of 193 to 140 gm., a 27% decrease in weight. Contrary to the results reported by Shaw and Greep, the hypophysectomized rats on diet #100 also showed a decrease in weight from an average of 201 gm. to 163 gm., a 19% decrease. It should be emphasized, however, that Shaw and Greep used rats of 28 days of age while these rats were from 6 to 7 months of age.

Table 1 shows that the hypophysectomized rats on the regular pellet diet developed the expected anemia. At 50 days the figures show a 27% decrease in erythrocyte count, 34% decrease in hematocrit, and a 30% decrease in hemoglobin. The rats on diet #100 also became anemic but not as severely so as those on the regular diet. The figures (50 days) show an 18% decrease in erythrocyte count, 20% decrease in hematocrit, and a 21% decrease in hemoglobin. The values for mean corpuscular diameter, mean

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[1] This work was done with the aid of a contract with the Office of Naval Research and the Atomic Energy Commission, Washington, D. C.

[2] Rockland Farms Complete Rat Diet.

[3] Diet #100 was prepared as follows:—Sucrose = 67 gm.; Casein = 24 gm. (to which was added 350 μg. thiamine hydrochloride, 350 μg. riboflavin, 350 μg. pyrixodine hydrochloride, 2.5 mg. nicotinic acid, 2.0 mg. calcium pantothenate, 100 mg. choline chloride, 100 mg. inositol, and 30 mg. para-aminobenzoic acid); Salts = 4 gm. (See J. Dental Research 26; 47, 1947); Corn oil = 5 gm. (to which was added 1.1 mg. beta-carotene, 300 I.U. irradiated ergosterol, 0.6 mg. 2-methyl-1 4-naphthoquinone, and 5.0 mg. alpha-tocopherol); Whole liver substance = 2 gm.; 1:20 liver concentrate powder = 2 gm.

TABLE 1. EFFECTS OF SHAW-GREEP DIET #100 ON THE ANEMIA WHICH IS INDUCED BY HYPOPHYSECTOMY IN ADULT FEMALE RATS

Day = Days of treatment; BW = Body weight; TRC = Total red cell count in millions per cu. mm.; Hemat = Hematocrit in per cent; HB = Hemoglobin in gm. per 100 cc; MCD = Mean corpuscular diameter in micra; MCV = Mean corpuscular volume in cu. microns; MCH = Mean corpuscular hemoglobin in micromicrograms; MCHC = Mean corpuscular hemoglobin concentration in per cent; ± = Standard deviation.

Day	BW	TRC	Hemat	HB	MCD	MCV	MCH	MCHC
				Normal Controls				
0	186 ±10.8	8.34 ±.31	43.8 ±1.22	13.9 ±.62	5.2 ±.13	52.4 ±1.20	16.7 ±.69	31.8 ±1.03
10	197 ±13.3	7.93 ±.60	42.5 ±2.52	13.6 ±.43	5.4 ±.14	53.8 ±3.15	17.1 ±.95	31.8 ±.55
20	207 ±16.5	8.23 ±.17	43.2 ±0.85	13.8 ±.36	5.5 ±.17	52.4 ±1.69	16.7 ±.64	31.9 ±.46
30	210 ±17.3	7.66 ±.50	43.4 ±1.26	13.6 ±.37	5.5 ±.11	56.8 ±2.25	17.8 ±1.09	31.3 ±.96
40	207 ±13.5	8.25 ±.60	44.1 ±1.50	13.9 ±.61	5.5 ±.14	53.4 ±3.30	16.8 ±.80	31.4 ±.58
50	208 ±21.6	8.25 ±.81	45.0 ±3.44	14.0 ±1.00	5.5 ±.20	54.6 ±1.94	17.0 ±.60	31.1 ±.41
				Hypophysectomized rats on regular stock pellet diet.				
0	193 ±12.8	7.86 ±.36	42.9 ±1.71	13.6 ±.69	5.5 ±.15	54.6 ±2.00	17.3 ±.67	31.7 ±.68
10	168 ±9.1	8.20 ±.52	44.7 ±3.40	14.6 ±1.12	5.6 ±.10	54.5 ±2.00	17.8 ±.77	32.6 ±.87
20	162 ±8.7	7.59 ±.66	40.9 ±1.60	13.0 ±.88	5.6 ±.14	52.5 ±2.40	17.1 ±.80	32.6 ±.72
30	151 ±9.0	6.84 ±.74	34.8 ±3.00	11.5 ±1.05	5.7 ±.14	51.0 ±2.21	16.9 ±.84	33.1 ±1.13
40	148 ±6.5	5.93 ±.60	30.6 ±2.51	10.2 ±.88	5.7 ±.16	51.8 ±.91	17.2 ±.90	33.2 ±.97
50	140 ±9.3	5.72 ±.44	28.0 ±1.87	9.5 ±.64	5.7 ±.14	49.0 ±2.88	16.6 ±.92	33.9 ±1.29
				Hypophysectomized rats on Shaw-Greep Diet #100				
0	201 ±11.8	8.33 ±.49	43.4 ±2.34	14.0 ±.72	5.3 ±.16	52.1 ±2.38	16.8 ±.78	32.2 ±.56
10	183 ±11.1	8.74 ±.66	46.6 ±2.94	14.7 ±.82	5.8 ±.17	53.4 ±1.88	16.8 ±.65	31.6 ±.90
20	171 ±19.9	8.68 ±.83	45.0 ±3.47	14.4 ±.97	5.5 ±.17	51.9 ±2.82	16.6 ±.82	32.0 ±.62
30	168 ±14.8	7.52 ±.38	38.9 ±2.71	12.7 ±.81	5.6 ±.10	51.6 ±3.15	16.9 ±.97	32.6 ±.61
40	164 ±11.7	7.16 ±.62	35.4 ±2.90	11.4 ±.73	5.6 ±.18	49.4 ±2.26	16.0 ±.93	32.3 ±1.21
50	163 ±13.0	6.84 ±.43	34.6 ±3.09	11.1 ±.72	5.6 ±.10	50.5 ±2.36	16.2 ±1.10	32.3 ±2.64

corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration are also given in the table.

In spite of the fact that the hypophysectomized rats on the prepared diet became anemic, they were more alert and seemed to be in a healthier condition. 36% of the rats on diet #100 died before the end of the experiment (50 days) while 47% of the rats on the regular pellet diet died before 50 days. In all fairness, however, it should be noted that the latter group were more anemic, a factor which may have contributed to their downfall.

The diet #100 used in this experiment was very generously provided by Dr. James H. Shaw and Dr. Roy O. Greep of the Harvard School of Dental Medicine.

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THE PRODUCTION OF PERMANENT DIABETES BY HIGHLY PURIFIED GROWTH HORMONE

The administration of highly purified growth hormone preparations has resulted in the production of temporary diabetes in cats (Cotes, Reid and Young, 1949), and in dogs (Campbell, Davidson, Snair and Lei, 1950). This suggested that the same material might produce permanent diabetes. The dogs used in our previous studies on the diabetogenic activity of various pituitary preparations were therefore again treated with a highly purified, crystalline growth hormone preparation (W.5). Permanent diabetes has been produced in two of them.

In testing pituitary preparations, temporary diabetes had been produced in these dogs previously on several occasions (the last preparation used being growth hormone W.4.P.), but no signs of diabetes had been apparent for 15 weeks prior to the present experiment. In this study, dog P. (15 kg.) received an average of 3.03 mg. of W.5 per kg. per day subcutaneously for 27 days. For 6 weeks following this treatment the average daily sugar excretion was 174 gm. Despite this great loss the dog remained in moderately good condition without any exogenous insulin. The diet, which consisted of lean meat, a prepared dry chow and sucrose, was increased in amount during this post-injection period until the daily intake was 1960 calories, derived from protein, fat and carbohydrate in the proportions of 35, 27 and 38% respectively.

Dog W. (11.3 kg.) was made diabetic by the administration of 3.5 mg.

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W.5 per kg. for 3 days. Following this treatment, severe diabetes persisted, and an average of 156 gm. sugar were excreted daily in the next 7 weeks. At the end of this time the dog was in a wasted and weak condition and it was necessary to administer insulin in order to save the animal. This treatment appears to be all that is required to maintain the dogs in excellent condition, though the effect of the insulin administration has been followed only up to 7 weeks. There has been no sign of decrease in the severity of the diabetes during the 10 and 14-week periods since the pituitary injections were stopped in dogs P. and W.

When preparation W.5 was administered to dog F., hyperglycemia, glucosuria and ketonuria occurred from the 4th to the 7th days inclusive, but then disappeared. From the 3rd day of treatment only part of the food was eaten. Since the food intake remained low and the signs of diabetes did not return, the injections were stopped after 17 days.

The crystalline growth preparation W.5 was prepared in the same way as was W.4, described previously, according to the methods of Wilhelmi, Fishman and Russell (1948). Its growth-promoting activity was comparable to that of the preparation (W.4.P.) used previously to produce temporary diabetes. It contained about 2% of ACTH when assayed by the "difference" method of Sayers, Sayers and Woodbury (1948). It is probable that this amount of ACTH was of little significance with respect to the production of permanent diabetes.

An electrophoretic analysis of W.4.P., used in the previous experiments, was carried out by Dr. A. E. Wilhelmi. The results of the analysis indicated that this preparation was at least 90% pure with respect to growth hormone.

It is not possible to estimate the degree to which the capacity of the beta cells of the islets of Langerhans to produce insulin had been affected by the tests of the diabetogenic activity of various anterior pituitary preparations prior to the present experiments. It would be preferable to demonstrate the production of permanent diabetes in dogs not used previously for such tests, but we have little doubt that this will be accomplished by the administration of the purified growth hormone preparation. It was evident that the dog W. was more sensitive to the diabetogenic effect of W.5 after the last test with the purified growth hormone preparation W.4.P; and dogs P. and F. also appeared to be more sensitive. The evidence available on dogs supports the observation of Reid (1949) on cats that the purified growth-promoting material increases sensitivity to subsequent treatment more than impure extracts do.

The production of permanent diabetes in these dogs is highly significant, both with respect to the identity of the diabetogenic material and the interpretation of its mode of action. It is intended to discuss these and other findings in more detail at a later date.

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